16 Cyanobacterial-Plant Symbioses

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Introduction

Cyanobacteria are unique in the wide range of symbiotic associations they form with eukaryotic hosts including plants, fungi, sponges, and protists (for reviews, see Adams 2000; Adams and Duggan 2012; Adams et al. 2012; Rai et al. 2000; Rai et al. 2002; Bergman et al. 2003, 2008). Cyanobacteria are photoautotrophs, and in many cases facultative heterotrophs and nitrogen fixers, and can provide nonphotosynthetic hosts with both nitrogen and carbon. Even if the benefit to the host is clear, that to the cyanobacteria is less obvious. They often receive carbon from photosynthetic hosts, but they are capable of carbon fixation themselves. Perhaps, in the enclosed environment provided by the host, a more likely advantage is protection from predation and from environmental extremes, such as high light intensity and desiccation.

The cyanobacterial symbionts of plants all possess at least two essential characteristics—the ability to differentiate heterocysts, which are specialized nitrogen-fixing cells (for reviews, see Adams and Duggan 1999; Zhang et al. 2006), and hormogonia, which are short, gliding filaments that lack heterocysts and provide a means of dispersal (Adams 2000; Meeks 2003, 2009; Gusev et al. 2002; Meeks et al. 2002; Meeks and Elhai 2002; Bergman et al. 2007). The hormogonia serve as the infective agents in most plant symbioses; some plants enhance their chances of infection by producing chemical signals that stimulate hormogonia formation and also chemoattractants that direct hormogonia into the plant tissue. Cyanobacteria are not restricted to the roots of plants but can infect thalli, stems, and leaves. The major hosts are bryophytes (see the section "Cyanobacterial Symbioses with Hornworts and Liverworts" in this chapter), the angiosperm *Gunnera* (see the section "Interactions in the *Nostoc*-*Gunnera* Symbiosis" in this chapter), the aquatic fern *Azolla* (see the section "The *Azolla* Symbiosis" in this chapter), fungi (forming lichens; see the section "Cyanolichens" in this chapter), the fungus *Geosiphon* (see the section "The *Geosiphon pyriformis*: *Nostoc* Endocyanosis and its Relationship to the Arbuscular Mycorrhiza (AM)" in this chapter), and cycads (see the section on "The Cycad Symbioses" in this chapter).

Cyanobacterial Symbioses with Hornworts and Liverworts

Bryophyte Symbioses

The division Bryophyta consists of the Hepaticae (liverworts), the Anthocerotae (hornworts), and the Musci (mosses), all of which are small, nonvascular terrestrial plants, some of which form epiphytic or endophytic associations with cyanobacteria, primarily of the genus *Nostoc* (Adams 2002a, b; Meeks 2003; Solheim et al. 2004; Adams et al. 2006, 2012; Adams and Duggan 2008; Bergman et al. 2007, 2008). Moss-associated cyanobacteria are mostly epiphytic (Solheim and Zielke 2002; Solheim et al. 2004; Gentili et al. 2005), apart from those found in two *Sphagnum* species in which the cyanobacteria are found in water-filled, hyaline (dead) cells, where they may be protected from the acidic bog environment (Solheim and Zielke 2002). Even these associations can be considered epiphytic as the hyaline cells are connected via pores to the outside environment. A wide range of cyanobacteria, including members of the non-heterocystous, filamentous genera *Phormidium* and *Oscillatoria* and even the unicellular *Microcystis*, have been found as moss epiphytes (Solheim et al. 2004), although members of the filamentous, heterocyst-producing genera *Nostoc*, *Stigonema*, and *Calothrix* are the most common (DeLuca et al. 2002, 2007; Gentili et al. 2005; Houle et al. 2006). These epiphytic associations will not be discussed further here, but they are of ecological importance as they are commonly the major source of combined nitrogen in ecosystems where mosses are abundant, such as northern hemisphere forests (Zielke et al. 2002, 2005; Solheim and Zielke 2002; Nilsson and Wardle 2005; DeLuca et al. 2008; see also Adams et al. 2012).

In their natural habitat, the liverworts and hornworts grow as a prostrate gametophyte thallus a few centimeters in length, attached to the substratum by primitive roots known as rhizoids. Mature symbiotic colonies can be seen as dark spots 0.5–1.0 mm in diameter within the plant tissue (*Fig. 16.1*). Of the more than 340 liverwort genera, only four are known to develop

Fig. 16.1
The liverwort *Blasia pusilla*, collected from the wild, showing the thick midribs of the thallus surrounded by the dark spots of *Nostoc* colonies (From Adams (2000), with permission)

associations with cyanobacteria: two (*Marchantia* and *Porella*) forming epiphytic associations and two (*Blasia* and *Cavicularia*) forming endophytic associations (Meeks 1990). Four of the six hornwort genera (*Anthoceros*, *Phaeoceros*, *Notothylas*, and *Dendroceros*) form endophytic associations (Meeks 1990). The epiphytic associations are more common than once thought but are poorly understood (Dalton and Chatfield 1985; Brasell et al. 1986), whereas the endophytic associations have been well studied because of the ease with which they can be grown in the laboratory. The hornworts *Anthoceros* and *Phaeoceros* and the liverwort *Blasia* can all be grown conveniently in shaken liquid culture (*Fig. 16.3b*), with or without their symbiotic partners, and can be readily reinfected with their original partner or with cyanobionts from *Gunnera*, cycads, lichens, and even some free-living strains (Enderlin and Meeks 1983; Meeks 1988, 1990, 2003; Kimura and Nakano 1990; Babic 1996; West and Adams 1997; Adams 2002a, b; Duckett et al. 2004; Adams and Duggan 2008; *Fig. 16.3c*).

The Symbionts

For a cyanobacterium to establish a successful plant symbiosis, it must possess at least two essential characteristics—the ability to differentiate both heterocysts, which are specialized nitrogen-fixing cells (for reviews, see Adams and Duggan 1999; Zhang et al. 2006), and hormogonia, which are short, gliding filaments that lack heterocysts and provide a means of dispersal (Campbell and Meeks 1989; Johansson and Bergman 1994; Bergman et al. 1996). Heterocysts fix dinitrogen for both partners, and the motile hormogonia, which are a transient phase of the life cycle, enable the otherwise immotile cyanobacterial filaments to gain entry to the plant host (see the section "Bryophyte Structures and Their Infection" in this chapter). The symbiotically competent cyanobacteria are hormogonia-forming strains of mostly the genus *Nostoc*, although *Calothrix* and *Chlorogloeopsis* strains have been shown to reconstitute the symbiosis with *Blasia* and *Phaeoceros* (West and Adams 1997). In the field, a single liverwort or hornwort thallus can become infected by many different *Nostoc* strains (West and Adams 1997; West et al. 1999; Costa et al. 2001; Rasmussen and Nilsson 2002; Adams and Duggan 2008).

Hormogonia differentiation is triggered by environmental stimuli, including the dilution of liquid cultures, or their transfer to solid medium or exposure to red light (Herdman and Rippka 1988; Tandeau de Marsac 1994). Their formation can also be triggered by exudates from plants such as *Anthoceros* (Campbell and Meeks 1989), *Blasia* (Knight and Adams 1996), *Gunnera* (Rasmussen et al. 1994), and wheat roots (Gantar et al. 1993; Knight and Adams 1996). The first 24 h of *Nostoc punctiforme* hormogonia development, induced by hormogonia-inducing factor (HIF, see section "Bryophyte Structures and Their Infection" in this chapter) or combined nitrogen starvation (Campbell et al. 2007, 2008), is characterized by many changes in gene expression, with the transcription of 944 genes upregulated and 856 downregulated (Campbell et al. 2007). The upregulated genes reflect the importance of signal sensing and chemotaxis because a majority of the encoded proteins are involved in signal transduction and transcriptional regulation, and others have putative roles in chemotaxis and pilus biogenesis (Meeks et al. 2001; Klint et al. 2006; Campbell et al. 2007).

Abundant type IV pili (Tfp) cover the surface of *Nostoc* hormogonia but are absent from vegetative cells (*Fig. 16.2*). In a wide range of bacteria, Tfp have roles in adhesion, motility, pathogenesis, and DNA uptake (Mattick 2002; Nudleman and Kaiser 2004; Burrows 2005). Both adhesion (to the plant surface) and motility (together with chemotaxis, to locate the host plant symbiotic structures) are likely to be essential factors in the successful infection of plants. Tfp are involved in motility in some unicellular cyanobacteria (Bhaya 2004) and may also have a role in the gliding of hormogonia, although this is so far unproven (Duggan et al. 2007). In *Nostoc punctiforme*, the mutation of genes such as *pilT* and *pilD*, thought to be involved in Tfp function, greatly reduces the infectivity of the mutant hormogonia in the liverwort *Blasia* (Duggan et al. 2007). However, it is not clear if this is due to loss of motility (and with it, chemotaxis) or interference with another potential function of the pili, such as recognition of, or adhesion to, the plant surface.

The ability of hormogonia to infect a particular host can be affected by subtle aspects of their behavior. For example, the infection frequency of *Nostoc punctiforme* hormogonia in the liverwort *Blasia* is influenced by mutations in *cyaC*, which encodes adenylate cyclase, the enzyme responsible for the

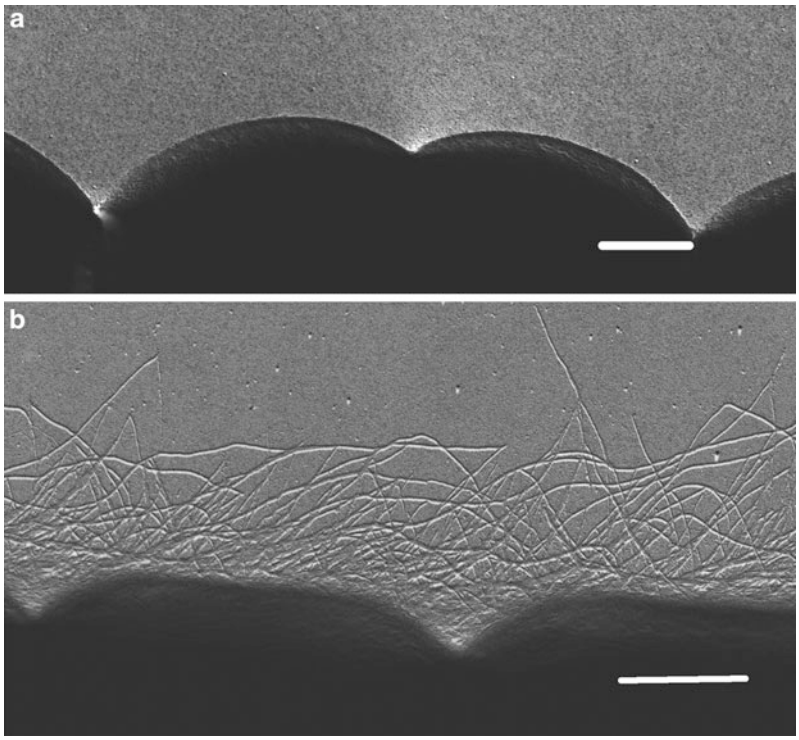


Fig. 16.2
Pili on the surface of *Nostoc punctiforme* hormogonia. Pili are absent from the cell surface of vegetative filaments (a) but are abundant on the surface of hormogonia (b). Scale bars represent 1 μm. For electron microscopy, platinum was evaporated onto the surface of each sample which was then viewed using a JEOL1200EX transmission electron microscope at 80 kV (From Duggan et al. (2007) with permission)

biosynthesis of the intracellular messenger cAMP, adenosine 3′, 5′-cyclic monophosphate (Adams and Duggan 2008; Chapman et al. 2008). However, mutation in two different domains of this multi-domain enzyme results in hormogonia with very different infection frequencies in *Blasia*, one having a three- to fourfold greater infection frequency than the wild type and the other showing a 75 % reduction in frequency compared with the wild type (Chapman et al. 2008). The explanation of these different infection phenotypes is not readily apparent, as both mutants have cellular cAMP levels 25 % of the wild type, and the mutant hormogonia, induced in the presence of *Blasia*, show no differences in their frequency, motility, or piliation.

Bryophyte Structures and Their Infection

In the bryophyte-cyanobacteria symbioses, the symbionts infect existing plant structures. In the liverwort *Blasia*, the cyanobacteria occupy roughly spherical structures, known as auricles, on the underside of the thallus (*Fig. 16.3c, d*). These develop from a three-celled mucilage hair that undergoes extensive elaboration (Renzaglia et al. 2000). The thallus of the hornworts *Anthoceros* and *Phaeoceros* is much thicker than that of *Blasia*, and the cyanobacteria are found in slime cavities, within the thallus, that open to the ventral surface via slit-like pores or mucilage clefts (*Fig. 16.3a*). The mucilage clefts, which resemble stomata but are not thought to be related (Villarreal and Renzaglia 2006), are formed by the separation of adjacent epidermal cells, and their formation is followed by the development of a slime cavity directly beneath the cleft (Renzaglia et al. 2000). *Blasia* auricles have two slime papillae, one of which (the inner slime papilla) partly fills the auricle cavity, whereas the other (the outer slime papilla) arises from the thallus adjacent to the auricle (*Fig. 16.3d*). In the hornwort *Leiosporoceros dussii* (*Fig. 16.4a*), the slime cavities take the form of elongated mucilage-filled "canals" (*Fig. 16.4b*) that result from the separation of plant cell walls along their middle lamellae and are connected to the outside by mucilage clefts (*Figs. 16.4c, d*) through which *Nostoc* can gain entry. Branching of the canals results in an integrated network, enabling the symbiont to invade the whole thallus (Villarreal and Renzaglia 2006). The cyanobacteria enter *Blasia* auricles, and presumably hornwort slime cavities, as hormogonia (see the preceding section "The Symbionts"), whereupon they lose motility and differentiate heterocysts (Kimura and Nakano 1990; Babic 1996).

Bryophyte-Cyanobacterium Signal Exchange

Anthoceros punctatus releases an unidentified, low-molecular-mass, heat-labile product that stimulates

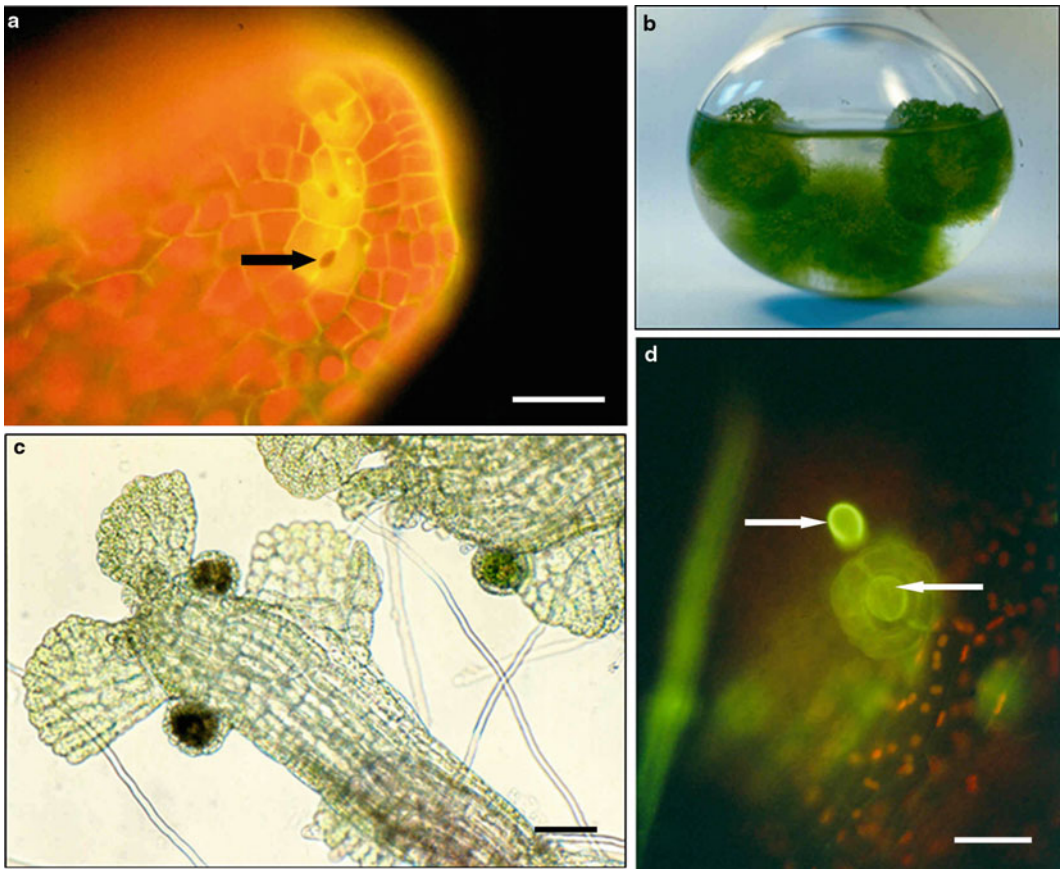


Fig. 16.3

The hornwort and liverwort symbioses. (a) Fluorescence micrograph of the hornwort *Phaeoceros* sp. stained with calcofluor. Hormogonia gain entry to the slime cavities within the thallus via slit-like entrances (one of which is *arrowed*). (b) View of the liverwort *Blasia pusilla* grown free of cyanobacteria in shaken liquid medium in an Erlenmeyer flask (viewed from *below*). (c) Liquid-grown *Blasia pusilla* infected in the laboratory with two different *Nostoc* strains, one *brown* pigmented (the two auricles to the *left*) and the other blue-green. (d) Fluorescence micrograph of an uninfected *Blasia* auricle stained with calcofluor. The auricle has one inner (*lower arrow*) and one outer (*upper arrow*) slime papilla. Bars 50 μm (Photographs (a) and (d) courtesy of S. Babic. (a, d) From Adams (2000) with permission; (b) from Adams (2002a) with permission; (d) from Adams and Duggan (1999) with permission)

hormogonia formation in *Nostoc* strains (Campbell and Meeks 1989). This hormogonia-inducing factor (HIF) seems to be produced as a result of nitrogen starvation, as it is not present when the hornwort is cultured in medium containing excess NH_4^+. Compounds with similar activity to HIF are found in *Gunnera* stem gland mucilage (Rasmussen et al. 1994), wheat root exudates (Gantar et al. 1993), and *Blasia* exudates (Babic 1996; Watts et al. 1999; Watts 2000). To attract hormogonia, a potential host must release a chemoattractant, such as that produced by the liverwort *Blasia* when nitrogen starved (Knight and Adams 1996; Watts 2000; Adams and Duggan 2008). However, hormogonia chemoattractants can also be produced by nonhost plants such as *Trifolium repens* (Nilsson et al. 2006) and germinating wheat seeds (Knight and Adams 1996; Watts 2000; Adams and Duggan 2008). Although the chemical identity of these chemoattractants is not known, they are thought to be sugar-based molecules (Watts 2000), and in keeping with this, simple sugars such as arabinose, glucose, and galactose are known to attract hormogonia (Nilsson et al. 2006).

As a symbiotic colony develops, filamentous protrusions grow from the host plant into the colony, possibly to enhance nutrient exchange between host and symbiont (see the section "Morphological Modifications to Bryophyte and Symbiont" in this chapter). What signal induces these changes in the host is not known; however, arabinogalactan proteins (AGPs) are released by many cyanobacteria (Bergman et al. 1996; Jackson et al. 2012), and such AGPs are thought to have important roles in plant growth and development (Pennell 1992). Liverworts also produce AGPs (Basile 1990), the inner and outer slime papillae of *Blasia* and the slime cavity of *Phaeoceros* staining with both Yariv reagent, which is specific for AGPs, and with anti-AGP monoclonal antibodies (Watts 2000; Jackson et al. 2012).

Another group of potential signaling molecules in cyanobacteria-plant symbioses is the flavonoids; these are secreted by legumes and are involved in the initial signaling in the symbiosis with *Rhizobium*, by binding to the transcriptional activator NodD (Fisher and Long 1992). Seed rinse from *Gunnera*, an angiosperm that forms symbiosis with *Nostoc*, can

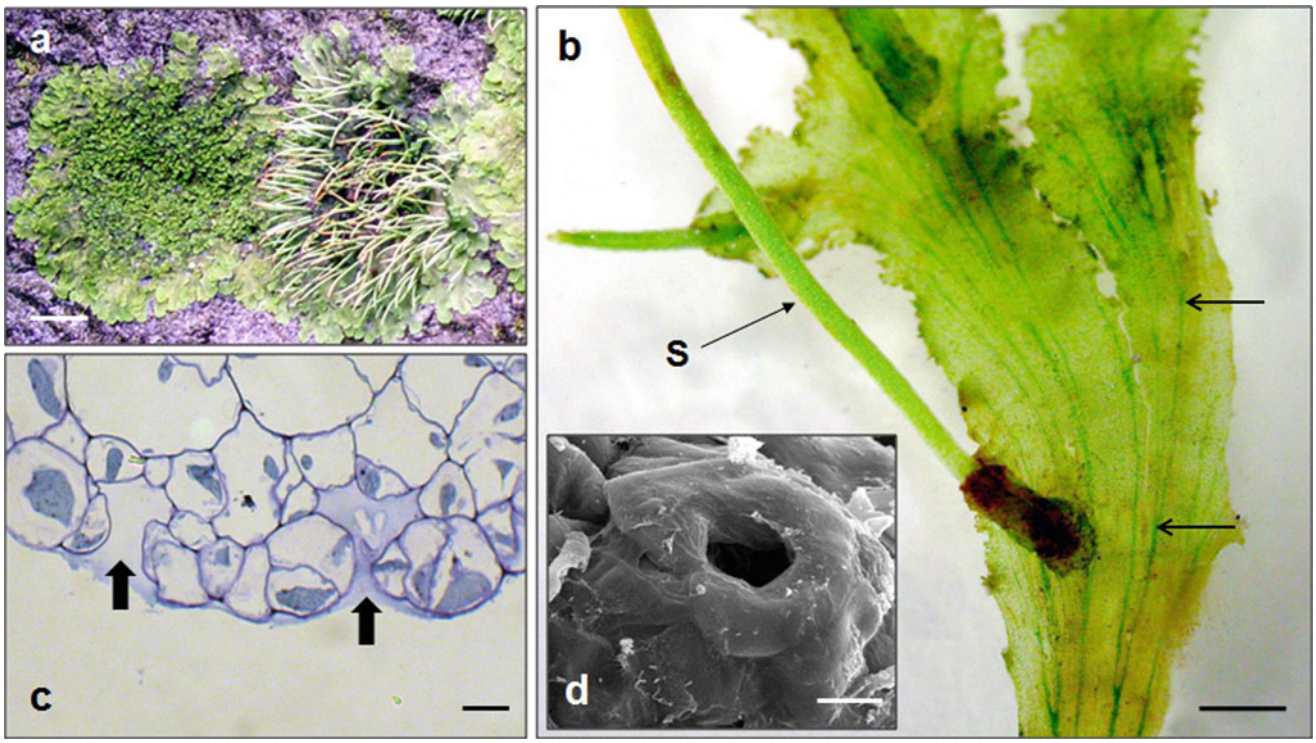


Fig. 16.4
The hornwort *Leiosporoceros dussii* with symbiotic *Nostoc*. (a) The young rosette to the *left* lacks the upright sporophytes that are abundant on the surface of the older thallus to the *right*. (b) The *Nostoc* colonies can be seen as long "strands" (some of which are *arrowed*) within the thallus, parallel to the main axis. S = sporophyte. (c) Light micrograph of a nearly transverse section of the mucilage clefts (*arrows*) that serve as the point of entry for cyanobacterial infection; the filaments of *Nostoc* subsequently spread through channels that result from the separation of hornwort cells along their middle lamellae. (d) Scanning electron micrograph of a mucilage cleft. Bars 10 mm in (a), 2 mm in (b), 15 μm in (c) and 20 μm in (d) (From Villarreal and Renzaglia (2006) with permission)

induce expression of *nod* genes in *Rhizobium* (Bergman et al. 1996; Rasmussen et al. 1996; Rai et al. 2000), and the flavonoid naringin induces expression of *hrmA* (see the sections "Cell Division Control and Hormogonia Formation" and "The *Hrm* Operon" in this chapter) in *Nostoc punctiforme* (Cohen and Yamasaki 2000). Expression of the *N. punctiforme hrmA* gene is also induced by a combination of components, including deoxyanthocyanins, found in extracts of the water fern *Azolla* which forms symbioses with *Anabaena* (Cohen et al. 2002).

The lectins are another group of signaling compounds of importance in bacterial symbioses. Although little is known about their potential involvement in cyanobacteria-plant symbioses, they are produced by the plant host in bryophyte and *Azolla* symbioses and bind to sugars on the surface of symbiotic *Nostoc* strains (Lehr et al. 2000; see also: Rai et al. 2000; Adams 2000; Rikkinen 2002; Adams et al. 2006, 2012). They have also been suggested to be involved in fungus-partner recognition in lichens (Lehr et al. 2000; Elifio et al. 2000; Rikkinen 2002; Legaz et al. 2004; Sacristan et al. 2006).

Host-Cyanobiont Interactions Post Infection

Cell Division Control and Hormogonia Formation

In symbiosis with *Anthoceros*, the doubling time of *Nostoc* can be 240 h, compared with 45 h in the free-living state (Meeks 1990). This slowed growth of the cyanobiont ensures that its growth rate matches that of the host plant. The mechanism of this growth control is unknown, but it seems not to be nitrogen limitation, even though the host takes most of the nitrogen fixed by its partner (see the section "Nitrogen Fixation and Transfer of Fixed Nitrogen" in this chapter).

As well as controlling the growth rate of the cyanobiont, the host must control hormogonia formation. Prior to infection, the host plant stimulates the development of hormogonia in potential partners by releasing HIF (see the section "Bryophyte-Cyanobacterium Signal Exchange" in this chapter). However, once infection has occurred, the plant must prevent hormogonia differentiation because hormogonia lack heterocysts and so cannot form a viable, nitrogen-fixing colony.

A hormogonia repressing factor (HRF), found in aqueous extracts of *Anthoceros* tissue (Cohen and Meeks 1997; Meeks 1998), inhibits HIF-induced hormogonia formation in wild-type *N. punctiforme*. The expression of two genes, *hrmA* and *hrmU*, is induced by HRF but not by HIF. These observations imply that the gene products of the *hrmUA* operon block hormogonium formation, perhaps by the production of an inhibitor or by the catabolism of an activator (Cohen and Meeks 1997; see the section "Genetic Analysis of the *Nostoc-Anthoceros* Association" in this chapter).

Morphological Modifications to Bryophyte and Symbiont

The cells of hornwort-associated *Nostoc* are often enlarged and show irregularities of shape compared with the same strains grown free living (Meeks and Elhai 2002). In free-living cyanobacteria, heterocyst frequency is typically 4–10 % of cells, whereas in symbiosis with hornworts and liverworts, frequencies are usually considerably higher (Adams 2000; Adams et al. 2012; *Table 16.1*). Although, in at least *Anthoceros*, some heterocysts seem to be senescent or dead (Meeks 1990), the increase in heterocyst frequency is still correlated with elevated rates of nitrogen fixation. Because heterocysts are unable to fix CO_2, this elevated heterocyst frequency results in a loss of CO_2-fixing capacity, which can be compensated by the supply of carbon skeletons by the host. In *Anthoceros*, and presumably all endophytic bryophyte associations, nitrogenase gene expression and heterocyst development in the symbiotically associated *Nostoc* appear to be controlled by plant signals and are independent of the nitrogen status of the cyanobiont (Campbell and Meeks 1992; Meeks 2003, 2009).

Morphological changes are also observed in the bryophyte following infection. In both *Blasia* and *Anthoceros*, branched, multicellular filaments grow from the wall of the symbiotic cavity and invade the colony, increasing the surface area of contact between the cyanobacteria and the bryophyte (Rodgers and Stewart 1974; Rodgers and Stewart 1977; Duckett et al. 1977; Renzaglia 1982; Kimura and Nakano 1990; Gorelova et al. 1996). In *Blasia*, these filaments are derived from the inner slime papilla and possess transfer cell morphology, implying an involvement in nutrient exchange. However, such wall ingrowths are not found in other hornworts, including *Leiosporoceros* (Villarreal and Renzaglia 2006).

Table 16.1
Summary of morphological and physiological changes in cyanobacteria symbiotically associated with hornworts and liverworts

Characteristic	Hornworts	Liverworts
Plant structure infected	Slime cavities	Auricles
Cyanobiont	*Nostoc*	*Nostoc* [a]
Location of cyanobiont	Intercellular	Intercellular
Heterocyst frequency (%)[b]	30–50	30–50
Nitrogenase specific activity[d]	443	n.d.
Glutamine synthetase:		
Amount of protein[c]	~86	n.d.
Specific activity[c]	~38	n.d.
Form of combined N released	NH_4^+	NH_4^+
Light-dependent CO_2 fixation (%)[d]	12	n.d.
RuBisCo:		
Amount of protein[d]	100	n.d.
Specific activity[d]	12	n.d.

Abbreviations: RuBisCo ribulose bisphosphate carboxylase/oxygenase, *n.d.*, not determined, though likely to be similar to hornwort data
[a]The symbionts are *Nostoc* spp. in almost all cases; there have been rare reports of *Calothrix* spp. as symbionts
[b]Heterocyst frequencies are expressed as a percentage of total cells. Typical values for free-living cyanobacteria are 4–10 %
[c]Values are for the symbiont as a percentage of the same cyanobacterium in the free-living state
[d]Values are expressed as a percentage of those for the free-living cyanobacteria
From Steinberg and Meeks (1989, 1991), Meeks (1990), Rai (1990), and Bergman et al. (1992a)

Nitrogen Fixation and Transfer of Fixed Nitrogen

The elevated rate of nitrogen (N_2) fixation in bryophyte-associated cyanobacteria broadly correlates with the increased heterocyst frequency in symbiosis (*Table 16.1*). The N_2 fixation rate of the *Anthoceros-Nostoc* association is 4- to 35-fold higher than that of free-living *Nostoc* (Steinberg and Meeks 1991). Such a high rate of N_2 fixation cannot be supported by the reduced photosynthetic capacity of the cyanobiont and must rely on reduced carbon derived from the plant.

Nitrogen fixed by the cyanobiont is released to the plant as ammonia (*Table 16.1*) in both *Anthoceros* (Rodgers and Stewart 1974; Stewart and Rogers 1977; Meeks et al. 1985a; Meeks et al. 1985b) and *Blasia* (Rodgers and Stewart 1974; Stewart and Rogers 1977), and initial uptake of the ammonia occurs via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway of the host (Meeks et al. 1983, 1985b; Meeks 1990; Rai 1990). In *Anthoceros*, the cyanobiont retains as little as 20 % of the nitrogen it fixes (Meeks et al. 1985a) yet shows no signs of nitrogen deprivation. Ammonia is released by the cyanobiont as a consequence of decreased activity of glutamine synthetase, the first enzyme in the GS-GOGAT pathway, which is the primary route of ammonia assimilation in cyanobacteria (Muro-Pastor et al. 2005; Flores and Herrero 2005). In *Anthoceros*, the decreased activity of GS appears to be the result of an undetermined posttranslational modification of the enzyme because the amount of GS protein differs little in filaments of free-living and symbiotically associated *Nostoc* (Joseph and Meeks 1987; Lee et al. 1988; Meeks 1990, 2003, 2009; Meeks and Elhai 2002; *Table 16.1*).

Carbon Dioxide Assimilation and Transfer of Carbon

The Calvin cycle is the primary route of CO_2 fixation in free-living and symbiotically associated cyanobacteria, with ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo) as the primary carboxylating enzyme (Tabita 1994). The rate of light-dependent CO_2 fixation in the *Nostoc* symbiont of *Anthoceros* immediately after its separation from symbiosis is eightfold lower than that of the same cyanobacterium in the free-living state (Steinberg and Meeks 1989; Meeks 1990; *Table 16.1*). However, the level of RuBisCo protein is similar in the two cases (Rai et al. 1989; Steinberg and Meeks 1989; Meeks 1990, 2003; Meeks and Elhai 2002), implying that activity is regulated by an unidentified posttranslational modification of the enzyme (Steinberg and Meeks 1989; Meeks 1990, 2003; Meeks and Elhai 2002). The cyanobiont therefore grows photoheterotrophically, receiving fixed carbon from its photosynthetic host, probably in the form of sucrose (Stewart and Rogers 1977; Steinberg and Meeks 1991). In at least *Anthoceros*, the presence of glycogen granules in the cells of symbiotically associated *Nostoc* implies that the symbiont is not starved of carbon (Meeks 1990).

Genetic Analysis of the *Nostoc-Anthoceros* Association

Meeks and coworkers have developed genetic techniques, including transposon mutagenesis, for the analysis of the symbiotically competent cyanobacterium *Nostoc punctiforme* strain ATCC 29133 (Cohen et al. 1994, 1998) and have used these techniques to identify a number of genes involved in the initial infection of *Anthoceros*. This has been aided by the availability of the complete genome sequence of *Nostoc punctiforme* {DOE Joint Genome Institute website} (see [{http://www.jgi.doe.gov}]).

The *hrm* Operon

In a transposon mutant of *Nostoc* 29133, characterized by an increased rate of initial infection of *Anthoceros* (Cohen and Meeks 1997; *Table 16.2*), Meeks et al. (1999) identified two open reading frames (ORFs), *hrmU* and *hrmA*, flanking the site of transposition (*Fig. 16.5*). *hrmA* has no significant similarity to sequences in major databases, whereas *hrmU* has similarity to the sequences of mannonate oxidoreductase genes and 2-keto-3-deoxygluconate dehydrogenase genes. Expression of *hrmUA* is induced by an aqueous extract of *A. punctatus* but not by the hormogonium-inducing factor, HIF. The aqueous extract appears to contain a hormogonium-repressing factor (HRF) because it suppresses HIF-induced hormogonia formation in the wild type but not in the mutant. Whereas HIF is released into the growth medium, HRF is probably released into the symbiotic cavity, suppressing further hormogonium formation and permitting heterocyst differentiation.

At the 5′ end of *hrmUA*, three other ORFs (*hrmI*, *hrmR*, and *hrmK*) are followed by two ORFs coding for unknown proteins, followed by *hrmE*, which has similarity to an aldehyde reductase (*Fig. 16.5*). HrmI shows similarity to uronate isomerase, HrmR to the LacI/GalR family of transcriptional

Table 16.2

Effect of insertion mutations on the symbiotic infectiveness (expressed in column two as the number of symbiotic colonies per unit of host tissue) and effectiveness (expressed in column three as acetylene reduction activity per g fresh weight of host tissue and in column four as acetylene reduction activity per symbiotic colony) of *Nostoc* 29133 strains in association with *Anthoceros punctatus*

Strain (gene)	Colonies per mg dry wt per μg Chl *a*	nmol C_2H_2 reduced per min per:		Gene induction factor(s)
		g of fresh weight	Colony ($\times 10^{-3}$)	
ATCC 29133 (WT)	0.21	6.3	12.4	n.d.
UCD 328 (*hrmA*)	1.6	6.1	8.6	HRF
UCD 398 (*sigH*)	1.2	8.0	10.1	HIF
UCD 400 (*tprN*)	0.49	10.4	6.7	HIF and HRF

Abbreviations: Chl *a*, chlorophyll *a*; HRF, aqueous extract of *A. punctatus* containing hormogonium-repressing factor identified as inducing the *hrm* operon; WT, wild type; HIF, exudate of *A. punctatus* containing hormogonium-inducing factor; and n.d., not determined (From Meeks et al. (1999). The standard deviations and number of replicates have been omitted for simplicity)

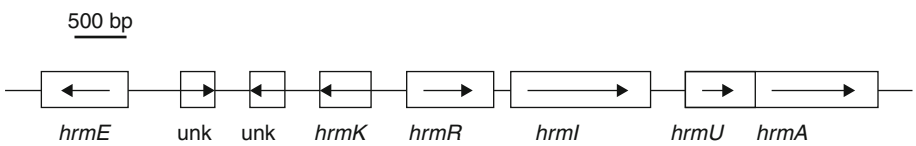


Fig. 16.5

Map of the open reading frames in the *hrm* locus of *Nostoc punctiforme*. The direction of transcription is indicated by the *arrows*. Unk are unknown proteins. Sizes are approximate (Adapted from Campbell et al. (2003))

repressors, and HrmK to gluconate kinases. HrmR is a DNA-binding protein that binds sugar ligands and represses transcription of *hrmR* and *hrmE* (Campbell et al. 2003). Galacturonate abolishes in vitro binding of HrmR to DNA, implying that the in vivo inducer may be a sugar molecule similar to or containing galacturonate. These observations led Meeks and coworkers to propose the following model for the way in which the HRF external signal is transduced into *Nostoc*. HRF enters the *Nostoc* cell and it, or a derivative similar to galacturonate, binds to HrmR, rendering it incapable of binding to the *hrmR* and *hrmE* operator regions; this derepresses transcription of these genes, leading to inhibition of hormogonia formation (Campbell et al. 2003).

sigH and *ctpH*

Mutation of the *Nostoc* 29133 *sigH* gene, which encodes an alternative RNA polymerase sigma subunit, produces no obvious phenotype in filaments grown in medium with or without combined nitrogen but results in an increased infection phenotype when they are cocultured with *A. punctatus* (Campbell et al. 1998; Meeks et al. 1999; Meeks and Elhai 2002; Meeks 2003; *Table 16.2*). Transcription of *sigH* is induced by *Anthoceros* HIF, but not by HRF, and *hrmA* transcription is not altered in a *sigH* mutant. Thus, although the *hrmA* and *sigH* mutants both have an increased infection phenotype, it seems likely that increased infection has a different basis in the two strains (Meeks et al. 1999).

The gene *ctpH* lies immediately 5′ of *sigH* and encodes a protein with significant similarity to carboxy-terminal proteases of the cyanobacterium *Synechocystis* PCC 6803 (Meeks et al. 1999). In *Synechocystis* 6803, this gene is required for processing the carboxy-terminal portion of the photosystem II D1 protein in the thylakoid lumen (Anbudurai et al. 1994). However, in *Nostoc* 29133, *ctpH* seems to have a different physiological role because it is not transcribed under vegetative growth conditions, but transcription is induced by *Anthoceros* HIF. The significance of this is not understood.

tprN

Lying 3′ of the gene *devR*, expression of which is essential for heterocyst maturation is the gene *tprN*, which encodes a protein with similarity to tetratricopeptide repeat proteins (Campbell et al. 1996). These proteins have been studied primarily in eukaryotes in which they are required for a variety of functions from cell cycle control to transcription repression and protein transport (Lamb et al. 1995). Inactivation of *tprN* in *Nostoc* 29133 has no apparent phenotypic effect in the free-living growth state, but the mutant infects *Anthoceros* at about twice the level of the wild type (*Table 16.2*). Transcription of *tprN* occurs during vegetative growth but increases in the presence of both HIF and HRF (Meeks et al. 1999). The significance of this in the infection process is not known.

ntcA, *hetR*, and *hetF*

Nostoc punctiforme (*Nostoc* 29133) strains unable to develop heterocysts because of mutations in either *hetR* or *hetF* can still infect *Anthoceros* at a frequency similar to that of the wild type, despite being incapable of forming a functional nitrogen-fixing symbiosis (Wong and Meeks 2002). *hetR* is thought to be the primary activator of heterocyst development (Wolk 2000; Golden and Yoon 2003; Zhang et al. 2006), and the HetF protein seems to be a positive activator of heterocyst differentiation, enhancing transcription of *hetR* and ensuring that HetR is localized to developing heterocysts (Wong and Meeks 2001).

In cyanobacteria, NtcA functions as a nitrogen-dependent global regulator (Herrero et al. 2004) and controls the transcription of a number of genes, including *hetR* (Fiedler et al. 2001; Herrero et al. 2001). The *Nostoc punctiforme ntcA* mutant, UCD 444, forms motile hormogonia with wild-type morphology but at only 5–15 % of the wild-type frequency (Wong and Meeks 2002). However, rather than infecting *Anthoceros* at a reduced frequency, as might be expected, the *ntcA* mutant fails to infect at all. This noninfective phenotype can be complemented with copies of *ntcA*.

Interactions in the *Nostoc-Gunnera* Symbiosis

The *Nostoc-Gunnera* Symbiosis

Although cyanobacterial-plant symbioses are the most widespread of the nitrogen-fixing symbioses, with hosts throughout the plant kingdom, those symbioses with angiosperms (flowering plants) are presently restricted to one monogeneric family, the Gunneraceae. This contrasts with the more recently evolved rhizobia- or *Frankia*-angiosperm symbioses, which involve a considerably wider angiosperm host range. The scarcity is also unexpected as angiosperms form the ecologically most successful plant division on earth, an area discussed in recent reviews by Osborne and Bergman (2009) and by Usher et al. (2007). In addition, cyanobacteria are globally widespread with a morphological variation surpassing most other prokaryotes. In spite of this, the cyanobacterial range is narrow, with only one cyanobacterial genus, *Nostoc*, functioning as microsymbiont in *Gunnera*. However, as the Gunneraceae is one of the oldest angiosperm families and with *Gunnera* and cyanobacterial fossils dating to some 90 million years ago (Ma) and three billion years ago (Ba), respectively, this symbiosis is likely to have persisted for a long time. Prior to the establishment of the *Nostoc-Gunnera* symbiosis, however, the same or a similar cyanobacterial genus may also have given rise to chloroplasts by entering some ancestral eukaryotic cell/organism. Indeed, the chloroplast genome of *Arabidopsis* is more similar to that of *Nostoc* than to the unicellular cyanobacteria tested (Martin et al. 2002; Deusch et al. 2008). This ancient endosymbiotic event (or series of events)

was the origin of all plants and algae and therefore totally revolutionized our biosphere and atmosphere (via oxygenic photosynthesis).

A Unique Endosymbiosis

Although the *Nostoc-Gunnera* symbiosis was first described by Reinke in 1873 (Reinke 1873), understanding of the infection mechanism in this unique angiosperm symbiosis is incomplete. In contrast to the other cyanobacterial-plant symbioses, the *Gunnera* symbiosis is exclusively intracellular. Still, being a facultative symbiosis, the cyanobiont is easily separated from the plant and may be grown independently, and the symbiosis can be reconstituted under laboratory conditions. This makes the *Nostoc-Gunnera* symbiosis an excellent model for identifying mechanisms involved in plant endosymbioses and indirectly in plastid evolution. Also, since it is the only plant symbiosis in which the cyanobacterium penetrates into the plant cells, the symbiotic development in *Gunnera* may have evolved further than that in all other plant symbioses in which the cyanobacterium remains extracellular.

The Symbionts

The genus *Gunnera* was named by C. von Linné in honor of the Norwegian bishop Gunnérus, a person Linné admired. The approximately 30–50 *Gunnera* species are mostly subtropical to tropical perennial herbs, the exception being the smallest, *G. herteri*, which is annual (Wanntorp et al. 2001; Osborne and Sprent 2002). The *Gunnera* plants are composed of large compound spikes and are rhizomatous, or more seldom stoloniferous, and have rhubarb-like leaves. Plant sizes vary considerably; some are gigantic and may be the largest herbs on earth, such as species in South America, Hawaii, and Asia, whereas others are small and creeping, such as the stoloniferous species in New Zealand. In nature, *Gunnera* spp. seem to be invariably infected by cyanobacteria (Wanntorp et al. 2001; Osborne and Sprent 2002).

Ever since the discovery of this peculiar symbiosis (Reinke 1873), cyanobacteria of the genus *Nostoc*, which are filamentous and differentiate heterocysts, have been identified as the sole cyanobionts (see Meeks et al. 2001; Meeks and Elhai 2002; Bergman et al. 2003). The phenotypic range of the cyanobiont of *Gunnera* is wide in terms of morphology, pigmentation, and colony shape and size, which is obvious when isolates are cultivated (Bergman et al. 1992b; Rasmussen and Nilsson 2002; Svenning et al. 2005; Papaefthimiou et al. 2008). A genotypic variation has also been verified using genetic fingerprinting of 45 cultured isolates originating from 11 *Gunnera* species (Nilsson et al. 2000; Rasmussen and Svenning 2001) and natural cyanobacteria freshly collected from different *Gunnera* growing in Chile (Guevara et al. 2002). One specific *Gunnera* plant may also occasionally be infected with more than one *Nostoc* strain (Nilsson et al. 2000), while no variation within one plant was found in cyanobionts of *Gunnera* spp. sampled from natural stands in Chile (Guevara et al. 2002) using the same fingerprinting technique. 16S rRNA analyses also demonstrate that all *Gunnera* isolates examined belong to the genus *Nostoc* (Rasmussen and Svenning 2001). Svenning et al. (2005) demonstrated that some cyanobacteria isolated from various *Gunnera* spp. may form a distinct clade (based on the complete 16S rDNA gene sequence) suggesting host specificity, although a few *Gunnera* isolates did not conform to this clade. Later it was suggested that most cyanobionts are affiliated to two clusters in which they are intermixed with free-living cyanobacteria (Papaefthimiou et al. 2008).

Specificity and Recognition

Although all *Nostoc* strains form hormogonia (the plant colonization units) per definition, still only certain strains of *Nostoc* are accepted as symbionts, which suggests the existence of other selective recognition mechanisms (see Rasmussen and Nilsson 2002). The intracellular position of the cyanobiont in *Gunnera* may also impose more severe restrictions on symbiotic partner recognition than in other intercellular, and possibly less intimate, plant symbioses. On the other hand, cyanobacterial isolates from cycads and bryophytes readily invade *Gunnera* cells and *vice versa*.

The Site of *Gunnera* Infection: The Gland

Infection occurs via a peculiar bright red gland (*Fig. 16.6*), already clearly visible at the developing cotyledon (see Bergman 2002; Bergman and Osborne 2002; Bergman et al. 2003; Chiu et al. 2005; Khamar et al. 2010). The development of the glands is a response to the nitrogen status of the *Gunnera* plant, and they only fully develop under nitrogen-deplete conditions. The plant

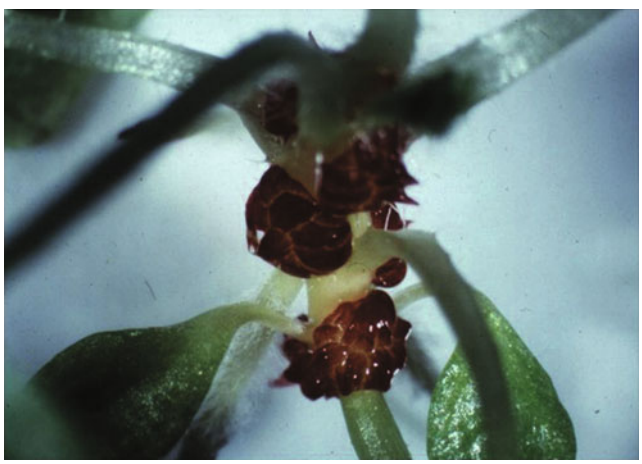


Fig. 16.6
***Gunnera* seedling with red stem glands out of which viscous mucilage is released**

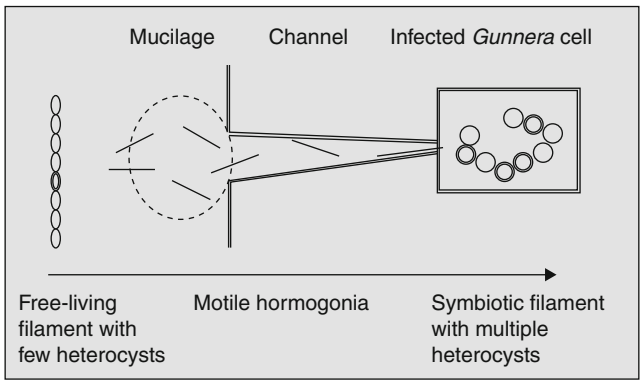


Fig. 16.7
Schematic illustration of the *Nostoc* infection process in *Gunnera*. Vegetative cells of *Nostoc* with heterocysts (5–10 %) are attracted to the mucilage pouring out of the *Gunnera* stem and stolon glands. The motile hormogonial stage is induced by the mucilage, and the cyanobacterium proceeds toward the interior of the gland. At the *bottom* of the channel, the cyanobacterial filaments penetrate the plant cell walls, and intact *Nostoc* filaments enter the *Gunnera* cells. After internalization, a cyanobacterial phenotype with larger cells and supernumerous heterocysts (up to 80 %) develops. The *arrow* indicates the direction of infection

hormone auxin is positively involved, potentially communicating the C:N status of the plant (Chiu et al. 2005; Khamar et al. 2010). Gland development is further accelerated under N-limited conditions if modest levels of sugars (0.5–1.0 % sucrose) are added (Chiu et al. 2005). This strengthens the significant role of the glands in plant nitrogen acquisition. Furthermore, high levels of carbohydrates (glucose and fructose), known to support symbiotic nitrogen fixation (Wouters et al. 2000), accumulate in the mature glands prior to the colonization by the cyanobacterium, while in *Nostoc*-colonized glands (in which nitrogen is replenished via nitrogen fixation) soluble sugar quantities are highly reduced (Khamar et al. 2010).

The glands secrete a carbohydrate-rich mucilage (*Fig. 16.7*) when non-infected, and new glands continuously develop at the base of each new leaf petiole, i.e., near the growing stem apices, which also become covered by the mucilage. Cyanobacteria-colonized glands are closed and do not release mucilage. Although root primordia were earlier suggested to be the point of entry (Schaede 1951), the present consensus is that glands are the sole cyanobacterial entry point (Silvester and McNamara 1976; Bonnett and Silvester 1981; Towata 1985; Johansson and Bergman 1992; Khamar et al. 2010). It has been proposed that these modified glands should be termed "nodules" (Silvester and McNamara 1976) and indeed a distinct and well-functioning symbiotic "organ," restricted in time and space, develops below the gland surface on colonization. Each gland, and possibly also each of the channels that penetrate into the gland (see the section "The Infection Process" in this chapter), functions as an independent colonization conduit, which would explain why several cyanobionts may be found inside one individual gland (Johansson and Bergman 1992; Nilsson et al. 2000).

The involvement of other microorganisms in the establishment of the *Gunnera* symbiosis, as proposed by Towata (1985), is not likely. This can be demonstrated by, for instance, reconstitution experiments under sterile laboratory conditions (Silvester and McNamara 1976; Johansson and Bergman 1992). In addition, some cells of the gland have heavy tannin depositions, which have been suggested to prevent the invasion of non-compatible or unwanted microorganisms (fungi and bacteria), which often reside together with cyanobacteria in the channel mucilage (Towata 1985).

The Infection Process

The focus has so far primarily been on morphological and adaptive changes in the cyanobiont. The plasticity of *Nostoc*, in this respect, is utilized by the plant throughout the colonization process and is likely a key factor contributing to its success as a *Gunnera* symbiont. A typical feature of the *Nostoc*-*Gunnera* symbioses is the tight regulation by the plant of cyanobacterial behavior such as cell division (considerably slowed down *in planta*), cell differentiation (the development of supernumerous heterocysts), and physiological performance (high nitrogen fixation rates).

Hormogonium Differentiation

A terrestrial cyanobacterium like *Nostoc* would (under normal free-living conditions) primarily occur as nonmotile, vegetative filaments with heterocysts at regular intervals (about 5–10 % of the total cell number; *Fig. 16.7*). On contact with *Gunnera*, the gland and the plant apex are, however, soon covered by a cyanobacterial "biofilm" composed of tightly packed hormogonia (Osborne et al. 1991; Johansson and Bergman 1992; Johansson and Bergman 1994; Chiu et al. 2005). Differentiation of these small-celled motile hormogonia is essential for the whole *Gunnera* colonization and cell penetration process; they act as a means for the cyanobacterium both to reach and to invade the *Gunnera* organ (the gland; *Fig. 16.7*). The mucilage has a pivotal role during this process (Rasmussen et al. 1994). It is composed of highly glycosylated arabinogalactan proteins (AGPs; Rasmussen et al. 1996) and stimulates not only growth but also hormogonium differentiation. A low-molecular-weight (<12 kDa), heat-labile protein, not yet characterized, which acts as hormogonium-inducing factor (HIF), has been identified in the mucilage (Rasmussen et al. 1994). In contrast, the soluble sugars of *Nostoc*-colonized glands inhibit hormogonium differentiation (Khamar et al. 2010). This is needed to stimulate heterocyst differentiation and nitrogen fixation, the "essence" of the symbiosis. Molecular mechanisms behind the induction of hormogonia and their differentiation are still largely unexplored.

Preliminary studies, using subtractive hybridization and proteomics (two-dimensional [2-D] gel electrophoresis coupled to mass spectrometry) of soluble *Nostoc* proteins treated with *Gunnera* mucilage show that the induction of hormogonium differentiation is also reflected in a differential expression of

genes and proteins, whose expression is either up- or downshifted or both. For instance, three mucilage-induced *hie* (host-induced expression) genes have been identified, including a putative precursor of a pheromone-like signaling peptide (HieA), an outer membrane or secreted glycoprotein (HieB), and a protein probably involved in adaptation to acidity (HieC; Liaimer et al. 2001). The latter may be important as the *Gunnera* mucilage has a pH of 4–5 (Rasmussen et al. 1994), a pH at the lower limit of the cyanobacterial tolerance range. Another set of proteins was also identified as being differentially expressed in hormogonia (Klint et al. 2006). These proteins, which were predominantly surface associated, may have roles in motility, recognition, adhesion, as well as in communication with host plants. The mucilage therefore appears to have important functions at earlier stages of the *Gunnera* infection process.

Entrance and Penetration

The *Gunnera* glands are composed of a set of up to nine papillae surrounding a central papilla (Johansson and Bergman 1992; Uheda and Silvester 2001; Chiu et al. 2005). Between the papillae, and leading into the stem tissue, are deep invaginations through which the mucilage is released. The hormogonia use these narrow channels to enter the dark interior of the *Gunnera* stems (*Fig. 16.7*). As this is against the normal positive phototactic behavior of *Nostoc*, a potent attractant must be released by the plant, possibly carried by the mucilage. Motility is crucial at this stage, as the direction of infection is opposite to that of the flow of mucilage. Upon reaching the bottom of the gland channels, the cyanobacterium penetrates the thin walls of smaller meristematic and dividing cells lining the channel (Silvester and McNamara 1976; Johansson and Bergman 1992; Johansson and Bergman 1994; Uheda and Silvester 2001). A delimited tissue of *Nostoc*-infected *Gunnera* cells is formed within a few days of inoculation. The mechanism(s) involved in the actual host cell penetration is still unknown, although Towata (1985) suggested the occurrence of pectolytic or cellulolytic activities in the mucilage of *G. kaalensis*. Also lining the channel are the thick-walled secretory cells releasing the mucilage (Towata 1985).

Signal Exchange Between the Cyanobacterium and the Host

Besides HIFs, the plant signals involved in hormogonium differentiation still await genetic identification and chemical characterization, as do the cellular response signaling cascades in *Nostoc*. In this context, a highly interesting question is whether the differentiation of hormogonia resulting from a biotic stimulus (such as *Gunnera* mucilage) triggers specific genes (such as those involved in "symbiotic competence") but not those triggered by any abiotic stimulus (such as red light). Also interesting are mechanisms involved in the initial rapid cell division and the machinery behind motility. All studies do, however, verify that the plant influences cyanobacterial morphology and behavior at all stages of the infection process and that this includes several fundamental cyanobacterial processes such as growth, cell division, cell differentiation, ammonia assimilation, and phototactic behavior. The question is whether this is triggered by plant compounds or by the environment within the plant. For instance, the symbiotic tissue is low in oxygen and light, which may have consequences for gene expression.

Another open question is to what extent the release of the auxin IAA (indole-3-acetic acid) by *Nostoc* (Sergeeva et al. 2002) acts as a signal or influences the development of the symbiotic *Gunnera* tissues. The influence of auxin has recently been stressed (Chiu et al. 2005). Indeed, cyanobacteria seem to have the potential to produce major phytohormones (Liaimer and Bergman 2003) and also to release "AGP-like" proteoglucans, which may also influence plant development (Bergman et al. 1996).

Host-Cyanobacterial Interactions Post Infection

Internalization of the cyanobiont elicits novel, dramatic modifications of cyanobacterial morphology and function (*Fig. 16.7*). Because hormogonia lack heterocysts, they are unable to fix nitrogen. Thus, the hormogonium stage is lethal under free-living conditions (unless combined nitrogen is available), and hence is, of necessity, transient. Redifferentiation into vegetative filaments with heterocysts occurs after 1–2 days. The maintenance of a continuous vegetative stage with heterocysts is a prerequisite for the symbiosis to persist as an efficient provider of combined nitrogen. Repression of hormogonium differentiation in *Gunnera* may be achieved by homologues to the hormogonium-repressing factor(s) (HRFs) identified in the bryophyte symbiosis (see Meeks and Elhai 2002). One component of this repression machinery may be the inhibitory effects on hormogonium development by the soluble sugars present in *Nostoc*-colonized glands (Khamar et al. 2010).

When inside the *Gunnera* cells, the cyanobacterial cells enlarge, and cell division is considerably restricted (Söderbäck and Bergman 1992). In addition, the filaments remain surrounded by the host cell plasmalemma through the pinocytosis process. This membrane, like the peribacteroid membrane in *Rhizobium*-legume symbioses, acts as the interface between the symbionts through which the exchange of metabolites takes place. The *Gunnera* cells eventually become filled with cyanobacterial filaments, which soon start to differentiate an abnormally high frequency of heterocysts (*Fig. 16.7*). Once infection is complete, the host must tightly control cyanobiont growth to avoid being outgrown, and this may explain the enlargement of cyanobacterial cells typical for the endosymbiotic stage (see Rai et al. 2000; Bergman 2002).

The dramatic morphological transitions seen in *Nostoc* on entering *Gunnera* cells are also reflected in the transcription of genes (and the corresponding proteins) related to heterocyst differentiation and nitrogen fixation (see Table 1 in Bergman 2002). For instance, the expression of the *hetR* gene (the master gene for heterocyst differentiation) correlates positively with the increase in heterocyst frequency, as does the expression of the

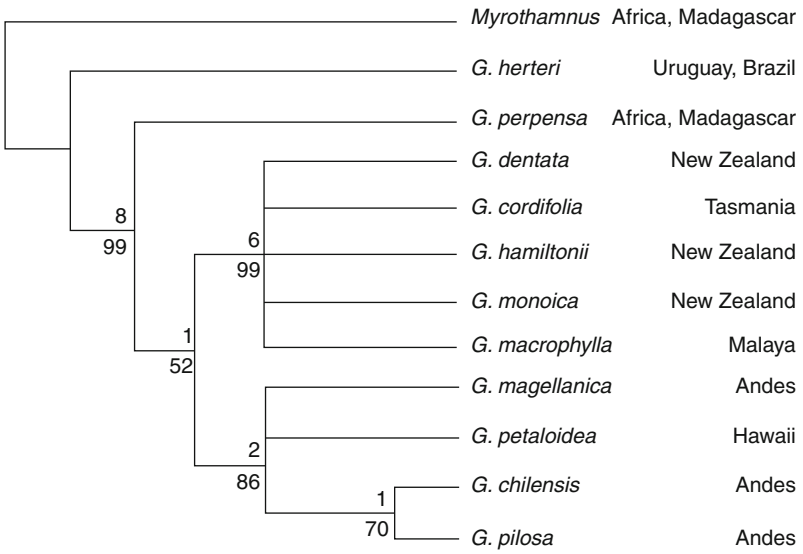


Fig. 16.8

Phylogram of 11 *Gunnera* species representing varying sizes and geographical origin. *Myrothamnus* is an African shrub-like plant growing in dry areas, as opposed to *Gunnera* species that prefer wet environments characterized by high humidity and high rainfall (From Wanntorp et al. (2001))

nitrogen-responsive transcription factor encoded by *ntcA*, whereas *nifH* expression is (as expected) already high, close to the growing apex. By contrast, the expression of the *glnB* gene, encoding the signal transduction protein P_{II}, decreases along the same symbiotic profile (Wang et al. 2004). The overexpression of both *hetR* and *ntcA* and the contrasting downregulation of *glnB* are features indicating important regulatory differences between the symbiotic and free-living life stages. Later, Ekman et al. (2006) identified a differential protein expression pattern in a cyanobacterium isolated from *Gunnera manicata* when using proteomic analysis. Changes were primarily related to cell envelope and membrane-associated proteins and to changes in cellular activities of C and N metabolism, including upregulation of nitrogenase and proteins of the oxidative pentose phosphate pathway and a downregulation of Calvin-Benson cycle enzymes. The significance of these findings in relation to cyanobacterial cell differentiation and the establishment and maintenance of an efficient nitrogen-fixing cyanobacterial-plant symbiosis now needs to be further explored.

Cross-sectioning of rhizomes of mature plants reveals the final outcome of the symbiosis: distinct and bright blue-green pigmented but restricted and delimited cyanobacterial colonies seen scattered in the rhizome or along the stolons of the smaller *Gunnera* plants (Osborne et al. 1991). However, the sites of infection comprise only a small proportion of the total plant biomass, particularly in the large *Gunnera* species.

Nitrogen Fixation and the Transfer of Nitrogen

As with most other plant symbioses, the main function of the cyanobacterium in *Gunnera* is to cover the total combined nitrogen requirement of the host via nitrogen fixation (Silvester and Smith 1969; Silvester 1976; Bonnett and Silvester 1981; Osborne et al. 1992; Khamar et al. 2010). The heterocysts act as the nitrogen-producing entities, holding all the nitrogenase (Söderbäck et al. 1990; Söderbäck 1992), and are capable of supporting the entire symbiosis with combined nitrogen. In addition, the cyanobacterium attains enhanced nitrogen fixation capacities compared to its free-living relatives (Silvester 1976; Bonnett and Silvester 1981). This may be related to the high heterocyst frequency or to an enhanced nitrogen starvation signal caused by the continuous N-drainage from the cyanobiont.

Up to 90 % of the nitrogen fixed is exported from the cyanobacterium to the host (Silvester et al. 1996). This is likely due to downregulation of glutamine synthetase protein levels, specifically in heterocysts, as well as other activities in symbiosis (Söderbäck 1992). As in most nitrogen-fixing plant symbioses, the nitrogen fixed is released primarily as NH_4^+ (Silvester et al. 1996). The *Nostoc*-infected *Gunnera* tissues are always well invested with vascular strands that facilitate exchange of metabolites such as nitrogen and carbohydrates (see ❯ *Fig. 16.8* in Bergman et al. 1992b). Multiple vascular strands (polystele) persist in *Gunnera*, which may be reminiscent of an aquatic ancestry (Osborne et al. 1991). Stock and Silvester (1994) showed, using pulse-chase labeling with ^{15}N, that the nitrogen fixed was efficiently transported from mature to young parts (with lower heterocyst frequencies) in *G. monoica* stolons and that N-translocation occurs via the phloem.

Carbon Assimilation and the Transfer of Fixed Carbon

As *Nostoc* inside the *Gunnera* cells is excluded from light, the host must supply the cyanobiont with fixed carbon via its

photosynthesis. Hence, the cyanobiont must adapt to a heterotrophic, or at least a mixotrophic, mode of life to generate enough reductant and ATP to support the demanding nitrogen fixation process (Söderbäck and Bergman 1992, 1993; Black et al. 2002; Khamar et al. 2010). Nevertheless, total pigment and ribulose-1,5-bisphosphate carboxylase levels remain constant along the developmental sequence, from young to old parts, although values decrease if related to cell volume as this increases in older cells (Söderbäck and Bergman 1992). The high frequency of heterocysts also drastically diminishes the number of vegetative cells, but the use of gas chromatography with mass spectrometry (GC-MS) has shown that still only the vegetative cells are actively taking up C (Black et al. 2002). Following ^{14}C translocation in *Nostoc*-infected *Gunnera* stolons reveals that the *Nostoc*-infected tissues at the apex of *G. magellanica* stolons are particularly efficient sinks for newly fixed plant carbon (Söderbäck and Bergman 1993). The phloem of *Gunnera* has the unusual capacity to contemporaneously transport N outward and C inward toward the symbiotic tissue (Stock and Silvester 1994). A tight interaction of nitrogen and carbon metabolism in the *Gunnera* symbioses is also suggested (see, e.g., Chiu et al. 2005; Khamar et al. 2010). *hetR* expression in symbiotically competent *Nostoc* (PCC 9229) is negligible in the absence of a carbon source in darkness but pronounced in the presence of fructose (Wouters et al. 2000).

Ecological Importance

Our understanding of the ecology and significance (e.g., as a nitrogen fixer) of this ancient plant and its cyanobiont is still rudimentary. For a detailed review of the ecology of *Gunnera*, the reader is referred to Osborne and Sprent (2002). The geographic range of *Gunnera* was considerably wider in the past when the climate was more favorable (Osborne et al. 1991; Osborne and Sprent 2002). Today, *Gunnera* typically grows in super-humid habitats and often at high elevations or on steep cliffs. The genus is found in all continents, except in Europe and polar regions (see Wanntorp et al. 2001; Osborne and Sprent 2002). Some large *Gunnera* species were introduced into Europe as ornamental plants at the end of the nineteenth century, and eventually some plants escaped and became invasive in, e.g., western Ireland, the Channel Islands, and the Azores (Osborne et al. 1991; Osborne and Sprent 2002).

The genetics (*rbcL* and *rps*16 introns) of *Gunnera* plant species have recently been analyzed (❯ *Fig. 16.8*). The large species in South America and Hawaii distinctly group together in one clade, the often smaller species of New Zealand and Southeast Asia group in another, while *G. perpensa* (the first *Gunnera* to be described by Linné) and *G. herteri* (with the smallest size) are sister groups, representing Africa and Brazil, respectively (Wanntorp et al. 2001).

Conclusions

From a cyanobacterial perspective, the *Nostoc*-*Gunnera* symbiosis may on the one hand seem wasteful; the cyanobiont merely functions as an N-producing entity with highly suppressed growth and is possibly deprived of producing a new generation of cyanobionts, being enclosed in tissues in a long-lived plant. On the other hand, it may be beneficial; the cyanobiont no doubt extends its ecological niche to also include symbiotically competent cells of an angiosperm. In this way, the cyanobacterium not only gains access to plant leaves and roots and their nutrient acquisition capacities but it also finds shelter from all possible predators, being the sole organism in this "golden cage."

The data obtained so far clearly show that cyanobacterial morphology and protein and gene expression patterns are drastically affected prior to, during, and after the establishment of the *Nostoc*-*Gunnera* symbiosis, although no symbiosis-specific genes and proteins, equivalent to the *nod* genes and Nod-factors in the *Rhizobium*-legume symbioses, have yet been discovered. However, it seems logical to assume that equally advanced molecular mechanisms must persist in a cyanobacterial-angiosperm endosymbiosis to generate this potentially very long-lived, well-coordinated, and successful interaction.

The *Azolla* Symbiosis

Introduction

Taxonomy and Distribution

The *Azolla* symbiosis is a mutualistic association between the aquatic fern *Azolla*, the filamentous, heterocystous, nitrogen-fixing cyanobacterium *Nostoc* (formerly classified as *Anabaena*), and endosymbiotic bacteria. The genus *Azolla* has been reported to contain seven extant species that are divided into two sections on the basis of spore morphology. Section *Azolla* (New World species) has included *A. caroliniana*, *A. mexicana*, *A. filiculoides*, *A. microphylla*, and *A. rubra*. However, the taxonomy of the New World species of *Azolla* has been the subject of much debate. In 2004, a comprehensive review of the literature was carried out along with original observations of type specimens using optical and scanning electron microscopy (Evrard and Van Hove 2004). This study confirmed the opinion of some that *A. caroliniana* and *A. microphylla* are synonyms of the previously described *A. filiculoides*. To clarify the taxonomic classification, the authors suggested the need to rehabilitate the Mettenius concept, and then according to the priority rule, the section *Azolla* species must be named *A. cristata* and *A. filiculoides*. Section *Rhizosperma* (Old World species) includes *A. pinnata* and *A. nilotica*. Geographically, *A. pinnata* is found in Australia, New Zealand, Japan, Asia, and Africa, and *A. nilotica* is primarily found in Africa (Saunders and Fowler 1993). Species from the section *Azolla* are more widely distributed around the world and are found in Europe, Asia, Africa, Australia, and America.

However, the distribution of *A. rubra* is restricted to New Zealand and Australia (Large and Braggins 1993). The distribution of some species has been impacted by human effects (Janes 1998a).

Morphology

The plant's shape, color, and size change significantly under different growth conditions (Janes 1998b). The rhizome is branched, bearing alternate leaves that are bilobed. The ventral lobe is transparent and serves to float the plant on the surface of the water, whereas the dorsal photosynthetic lobe contains a leaf cavity in which the symbionts are found. The roots are adventitious. The shedding of roots and branches is related to environmental and physiological factors and enables the plant to reproduce via vegetative fragmentation. Factors affecting the growth of *Azolla* include genotype, temperature, light (intensity, quality, and photoperiod), water chemistry (including pH, salinity, and nutrients), and influence of pests and diseases (see Singh and Singh 1997).

General Characteristics

The association has been most frequently used as an alternative nitrogen fertilizer in rice fields, as well as a supplemental animal fodder. *Azolla* provides the cyanobiont with nutrients, including fixed carbon, and the cyanobiont provides the host with combined nitrogen (via nitrogen fixation). The exact role of the endosymbiotic bacteria in the association remains unclear; however, some possible functions have been suggested.

The Symbionts

Cyanobacterial Symbionts

Identification

The filamentous, heterocystous, nitrogen-fixing cyanobacterial symbionts in the *Azolla* association have been extensively studied using both traditional and modern molecular techniques. In addition to characterization of the cyanobionts after they have been directly extracted from the association, there have been a number of studies in which cyanobacteria were isolated and cultured, in attempts to study the cyanobacterial symbionts in a free-living state. While numerous researchers have reported success in isolation and cultivation of the symbiotically associated cyanobacteria (Newton and Herman 1979; Tel-Or et al. 1983; Gebhardt and Nierzwicki-Bauer 1991; see Braun-Howland and Nierzwicki-Bauer 1990), molecular studies (primarily based on restriction fragment length polymorphism [RFLP] analyses) have indicated that none of the isolates represent the major cyanobacterial symbionts in the association (Gebhardt and Nierzwicki-Bauer 1991). Though not conclusively demonstrated, major as well as some minor cyanobacteria may be present in the association, with the more readily cultured cyanobacteria representing minor symbionts (Gebhardt and Nierzwicki-Bauer 1991). The other possible explanation is that the isolates presumably obtained from the association are actually epiphytes. However, recent studies of the genetic diversity of cultured cyanobionts of diverse species of *Azolla* revealed a genetic distinctness of the cultured *Azolla* cyanobionts as compared to free-living cyanobacterial strains of the genera *Anabaena* and *Nostoc* and symbiotic *Nostoc* strains from *Anthoceros*, *Cycas*, and *Gunnera* (Sood et al. 2008). These findings support the coexistence of minor species rather than epiphytes. Regardless, based on molecular studies, it has not been demonstrated that the major cyanobacterial symbiont from the association can be cultured in a free-living state. In fact, with the genome sequencing of the major cyanobiont of an *A. filiculoides* strain, there is now strong evidence (described below) that there has been ongoing selective streamlining of the cyanobiont genome which has resulted in an organism devoted to nitrogen fixation and devoid of autonomous growth (Ran et al. 2010).

Given the challenge of studying the cyanobacterial symbionts in a free-living state, direct molecular studies have been used for accurate identification. Restriction fragment length polymorphism (RFLP) analyses (Gebhardt and Nierzwicki-Bauer 1991), polymerase chain reaction (PCR) fingerprinting (Zheng et al. 1999), random amplified polymorphic DNAs (RAPDs; Van Coppenolle et al. 1995), as well as fluorescence in situ hybridizations (FISH) (Bushnell 1998) have been used to examine the identity of the symbiotic cyanobacteria. Regardless of the approach used, the cyanobiont referred to as "*Anabaena azollae*" has in most instances been described as being somewhat related to *Anabaena* or *Nostoc* (Plazinski et al. 1990b; Gebhardt and Nierzwicki-Bauer 1991). A study (Baker et al. 2003) using comparisons of sequences of the phycocyanin intergenic spacer and a fragment of the 16S rRNA gene places the *Azolla* cyanobiont in the order Nostocales but in a separate group from *Anabaena* or *Nostoc*. Additionally, near full-length (1,500 bp) 16S rRNA sequencing and phylogenetic analysis of major cyanobionts from a variety of *Azolla* species yielded similar results (Milano 2003). In 1989, Komarek and Anagnostidis placed the *Azolla* cyanobiont in a revised genus named "*Trichormus*" on the basis of morphology (Komarek and Anagnostidis 1989). This is not inconsistent with the most recent molecular-based findings.

Recently, the genome sequencing of a cyanobacterium from *Azolla filiculoides* leaf cavities has provided the most comprehensive information on its identity (Ran et al. 2010). Surprisingly, the phylogenetic analysis places the cyanobiont (*Nostoc azollae* 0708) most closely with *Raphidiopsis brookii* D9 and *Cylindrospermopsis raciborskii* CS 505, the two multicellular cyanobacteria with the smallest known genomes (Stucken et al. 2010). However, it shares the highest number of protein groups with *Nostoc* sp. PCC 7120, *Anabaena variabilis* ATCC 29413, and *N. punctiforme* PCC 73102 (Ran et al. 2010).

The taxonomy of the cyanobionts is generally in agreement with the taxonomy of the host plant (Plazinski 1990; Van Coppenolle et al. 1993; Zheng et al. 1999). These findings, taken in conjunction with the continuous maintenance of the symbiosis throughout the life cycle of the plant (see the section "The Infection Process" below), suggest coevolution of the cyanobionts and the host plant.

Developmental Profile Along the Main Stem Axis

The growth of the endophyte is coordinated with the growth of the plant. In the apical meristem and younger leaves, the cyanobacterial vegetative cells are smaller than in older leaves and undergo frequent cell divisions. Increases in leaf age are accompanied by a decrease in cell division and increased size of the cyanobacterial vegetative cells, as well as increased heterocyst frequencies (Hill 1975). The number of heterocysts and the nitrogen fixation rates vary in leaves of different ages, as well as in different *Azolla* species (Hill 1977). Heterocyst frequencies can reach up to 20–30 % of the cells within a filament in the symbiotically associated cyanobiont. These are much higher than the typical 10 % heterocyst frequency in free-living *Anabaena/Nostoc* species.

Bacterial Symbionts

The presence of bacteria residing within the leaf cavity of *Azolla* has been recognized for many years (Carrapico 1991), yet still unclear is the specific function(s) of most of the bacteria in this symbiosis. Initial attempts to study the symbiotic bacteria employed traditional microbiological, biochemical, and physiological techniques for the identification of bacteria isolated from a variety of *Azolla* species. Utilizing these approaches, there were many reports of *Arthrobacter* spp. (most frequently *A. globiformis*) occurring in symbiotic association with *Azolla* (Gates et al. 1980; Wallace and Gates 1986; Forni et al. 1989, 1990; Nierzwicki-Bauer and Aulfinger 1991; Shannon et al. 1993). *Agrobacterium* has also been reported to be isolated from different *Azolla* species (Plazinski et al. 1990a; Shannon et al. 1993; Serrano et al. 1999). Other bacteria, such as *Staphylococcus* sp., *Rhodococcus* spp., *Corynebacterium jeikeium*, and *Weeksella zoohelcum*, were identified by BIOLOG and API tests as being in association with *Azolla* (Serrano et al. 1999). A detailed review of the identification of bacteria isolated from *Azolla* species is provided in Lechno-Yossef and Nierzwicki-Bauer (2002). Molecular techniques, in particular 16S rDNA gene amplification, cloning, screening, sequencing, and phylogenetic analysis, have provided more detailed information on the identity of the symbiotic bacteria (Lechno-Yossef 2002; Milano 2003). In the accessions studied, sequence similarity found that the most abundant bacterial symbionts in *A. caroliniana* and *A. filiculoides* were *Frateuria aurantia* and *Agrobacterium albertimagni* and in *A. mexicana*, *Agrobacterium tumefaciens* (Lechno-Yossef 2002). More recent research studying the endophytic bacteria within *A. microphylla* using PCR-DDGE and electron microscopy revealed a complex and divergent bacterial community with *Bacillus cereus* as the dominant species (Zheng et al. 2008).

Host Structures and the Infection Process

The Leaf Cavity

In the association, the symbionts reside in a leaf cavity, an extracellular compartment in the dorsal lobe of the leaf. In mature leaves, the symbionts (cyanobacteria and bacteria) are located in the periphery of the leaf cavity in mucilaginous material between internal (Nierzwicki-Bauer et al. 1989) and external envelopes (Uheda and Kitoh 1991). Electron microscopic analysis combined with specific staining showed that the inner envelope does not have a tripartite structure typical of a membrane and is rich in lipids (Nierzwicki-Bauer et al. 1989). The external three-layered envelope is believed to contain cutinic and suberic substances, as revealed by response to chemical treatments of degradation using hot alkali methanol (de Roissart et al. 1994).

The adaxial epidermis of the leaf cavity contains a pore that is surrounded by two cell layers (Veys et al. 1999, 2000). One layer inside the pore is composed of teat-shaped cells that are extended from the adaxial epidermis. The other layer corresponds to the inner epidermis, which lines the inside of the cavity. Three to four tiers of teat cells form a cone-like pore with an average diameter at the base of 80 μm. The pore opening is larger in younger leaves, and the morphology of the teat cells suggests that their function is as a physical barrier to prevent particles and organisms from entering the cavity and the symbionts from exiting (Veys et al. 2002).

The Infection Process

Azolla is a heterosporous water fern that is capable of both sexual and asexual reproduction. Unlike any of the other cyanobacterial symbioses, the host is in continual association with the symbionts, making this the only known permanent symbiosis. Thus, rather than reinfect *Azolla*, the symbionts retain coordinated growth in association with the host throughout its life cycle. Descriptions of the processes involved in maintaining the continual association during sexual and asexual reproduction are described briefly below.

Sexual Reproduction

Sporulation is the sexual reproduction process in *Azolla*. During sexual reproduction, the host produces both mega- and microsporocarps. The partitioning of the cyanobacterial filaments into the developing sporocarps and the reestablishment of the symbiosis following embryogenesis were originally described for *A. mexicana* (Perkins and Peters 1993; Peters and Perkins 1993). The symbionts that are used as inoculum to the

developing sporocarps come from the dorsal lobe of the same leaf in which the sporocarps are developing. Recently, a comprehensive study of cellular responses in the cyanobacterial symbionts during its vertical transfer via megasporocarps between plant generations in the *A. microphylla* symbiosis was reported (Zheng et al. 2009). During colonization of the megasporocarp, the cyanobacterium entered through pores at the top of the indusium as motile hormogonium filaments. Subsequently, the cells differentiated into akinetes in a synchronized manner. Also discovered was that this process was accompanied by cytoplasmic reorganizations within the cyanobionts and the release of numerous membrane vesicles, most of which contained DNA, and the formation of a highly structured biofilm (Zheng et al. 2009). These data revealed complex adaptations in the cyanobacterium during transition between plant generations that merit further investigation.

The cyanobiont akinetes (which function as spores) and the bacterial symbionts (which do not always show ultrastructural characteristics of spore envelopes; Aulfinger et al. 1991) found in the megasporocarps are transferred to the developing spores and sporelings. After separation of the megasporocarp from the plant, part of the indusium is shed, and the proximal half becomes the indusium cap. The symbionts reside in a space called "the inoculation chamber" (Peters and Perkins 1993), located between the indusium cap and the apical membrane of the megasporocarp. Following fertilization and the beginning of embryogenesis, the symbionts resume metabolic activity. With the assistance of cotyledonary hairs, the symbionts are introduced into the embryonic leaf before it displaces the indusium cap (Peters and Perkins 1993). Leaves, which grow from the meristem, are initially unlobed but contain a structure similar to the leaf cavity that contains the symbionts. As the frondling continues to grow, the symbionts are distributed into the developing leaf cavities by a mechanism similar to the transfer mechanism used during asexual reproduction via vegetative fragmentation (see next section).

Asexual Reproduction

The main form of reproduction in *Azolla* is vegetative fragmentation. The apical meristem of each branch contains a colony of undifferentiated cyanobacterial cells. Cyanobacterial filaments from the apical colony are introduced into the leaf primordium before the development of the leaf and leaf cavity are complete. The partitioning of the endophytes into the developing leaves is facilitated by entanglement around primary branched hair (PBH) cells of *Azolla* (Calvert and Peters 1981). The leaf cavity starts to develop and engulf the cyanobacterial colony in the fourth or fifth leaf along the stem axis. In this way, symbionts are inoculated into every leaf cavity that is formed. The development of the leaf cavity is also accompanied by the formation of simple hair cells by *Azolla* (Peters and Calvert 1983).

"Artificial" In Vitro Infection of Cyanobacteria

In sporulating *Azolla*, sexual hybridization between different *Azolla* species, as well as the formation of new combinations of *Azolla* and *Nostoc*, has been somewhat successful (Watanabe 1994; Watanabe and Van Hove 1996). For example, *Nostoc* from *A. microphylla* (MI4031) was successfully introduced into *A. filiculoides* (FI1034) by exchange of the indusium cap of the megaspore (Lin et al. 1989). Successful sexual hybridizations between *A. microphylla* (megasporocarp) and *A. filiculoides* (microsporocarp; Wei et al. 1988; Do et al. 1989), between *A. filiculoides* (megasporocarp) and *A. microphylla* (microsporocarp; Watanabe et al. 1993), and between *A. mexicana* and *A. microphylla* (Zimmerman et al. 1991) have also been reported. The key to these successes has been having the cyanobacteria at the appropriate stage of development (during akinete germination and vegetative cell growth) that mimics what naturally occurs in situ.

Host-Symbiont Signal Exchange

The recognition between *Azolla* and *Nostoc azollae* is facilitated by lectins in both the plant (Mellor et al. 1981) and the cyanobionts (Kobiler et al. 1981, 1982). Additionally, bacteria isolated from *A. pinnata* and *A. filiculoides* have been shown to contain lectins (Serrano et al. 1999). The presence of Rhizobiaceae symbionts in association with different *Azolla* species and cultures examined would suggest that this group of bacteria has a role in the symbiosis. Plazinski et al. (1991) showed that the *nodL* and *nodABC* genes gave hybridization signals to a plasmid and the chromosome of the isolate AFSR-1 from *A. filiculoides*. These authors suggest that the *nod* genes, if active in the bacterial symbionts of *Azolla*, play a regulatory role in the development of the symbiosis or in the maintenance of bacterial association with the plant.

Host-Cyanobiont Interactions Post Infection

Morphological Modifications to Host and Cyanobacteria

The leaf cavity and inner and external envelopes of *Azolla* do not appear to be present only when it is symbiotically associated with the cyanobionts. These structures are present in both *Nostoc*-free and *Nostoc*-containing *Azolla* (Nierzwicki-Bauer et al. 1989). This evidence excludes the involvement of the cyanobionts in the formation of these structures. However, given that the cyanobiont-free plants examined still contained symbiotic bacteria, a possible role of bacteria in the synthesis of the leaf cavity envelopes cannot yet be excluded.

Nitrogen Fixation and Transfer of Fixed Nitrogen

Nitrogen fixation is carried out by the heterocysts of the cyanobiont. In leaf cavities of different ages along the stem axis of *Azolla*, the heterocyst frequencies and nitrogen fixation

rates vary. Nitrogen fixation, as determined by the acetylene reduction assay, occurs in the apical (younger) leaves but not in the stem apex, increases and reaches a peak in leaves of middle age, and then decreases in the older leaves (Canini et al. 1990). Ammonium, the product of nitrogen fixation, is released from heterocysts and assimilated by *Azolla* into glutamate using the glutamine synthetase (GS)-glutamate synthase (GOGAT) system (Peters and Calvert 1983). Nitrogenous compounds in the form of glutamate, glutamine, ammonia, and other glutamate derivatives are transferred from the mature leaf cavities to the stem apex (Peters et al. 1985). In *Nostoc azollae*, the activity and protein content of GS are only 5–10 % of that of free-living *Anabaena* (Orr and Haselkorn 1982). However, the nitrogen fixation activity is much higher because of the increased number of heterocysts. Some of the bacteria found in this association can fix nitrogen. Immunoelectron microscopy studies using antibodies against the Fe and FeMo protein subunits of nitrogenase revealed that a subset of the bacteria in the *A. caroliniana* and *A. filiculoides* associations contained these nitrogenase subunits (Lindblad et al. 1991). The potential nitrogen-fixing contribution of the bacteria in the association separate from that of cyanobacterial symbionts could not be measured because they coexist in the leaf cavities and, once removed, are likely to have altered capabilities.

Carbon Assimilation and Transfer of Fixed Carbon

The cyanobiont, *Nostoc azollae*, has photosynthetic capabilities; however, in the symbiotic state, it is believed to contribute less than 5 % of the total CO_2 fixed in the association (Kaplan and Peters 1988). Pulse-chase studies have shown that sucrose from the plant is supplied to and accumulated by the cyanobiont (Peters et al. 1985). Simple hair cells of *Azolla* are involved in the transport of sugars from the photosynthetic mesophyll cells to the leaf cavity. Simple hair cells have ATPase activity in their plasmalemma and some accumulation of starch in their chloroplasts (which do not possess ribulose 1, 5 bisphosphate carboxylase, RuBisCO), suggesting active transfer of sugars from the simple hairs to the leaf cavity (Carrapico and Tavares 1989). Additionally, primary branched hair cells, having the morphology of a transfer cell, are believed to be involved in nutrient transfer from the plant to the cyanobiont(s) (Peters et al. 1985).

Ecological Importance: Friend or Foe?

The *Azolla* symbiosis is of tremendous ecological importance, having both positive and negative impacts. On the positive side, the association has been extensively used as a biofertilizer, providing a source of combined nitrogen in the form of ammonium, thereby reducing or eliminating the need for the addition of chemical fertilizers. This role has been most extensively used in conjunction with rice paddies or fertilization of fields. The growth of *Azolla* into thick mats also makes it effective in suppressing weed growth. Owing to its high protein content, *Azolla* is used as a fodder for sheep, pigs, ducks, etc. The ability of *Azolla* to remove nitrates and phosphorous from water has resulted in improvement of water quality. Additionally, *Azolla* has been used to remove heavy metals from water. Ten useful characteristics attributed to this association have been described (Van Hove and Lejeune 1996; Lejeune et al. 1999), with the capacity to fix atmospheric nitrogen, high productivity, high protein content, and a depressive influence on both aquatic weeds and NH_3 volatilization being considered unquestionably useful.

The same characteristic feature that makes *Azolla* useful for weed suppression and biofertilization of fields (namely, the ability to grow in thick mats) also results in a number of negative ecological impacts. For example, growth of *Azolla* mats in streams in Zimbabwe has been shown to have a negative impact on animal biodiversity (Gratwicke and Marshall 2001). In many regions where *Azolla* is an invasive species, it has overgrown many native species. In efforts to control *Azolla* growth, biological controls such as the introduction of a frond-feeding weevil (McConnachie et al. 2004) or the flea beetle (Hill and Oberholzer 2002) are being explored. Thus, the overall ecological impact of the *Azolla* association continues to expand and may reach even to Mars, since *Azolla* is currently being used in studies examining possible bioregenerative life support on Mars (http://www.highmars.org/niac/niac04.html, The Caves of Mars Project website).

Cyanobionts Becoming a Nitrogen-Fixing "Organelle"?

The most important recent advancements related to the *Azolla* symbiosis have been proteomic (Ekman et al. 2008) and genomic analyses (Ran et al. 2010) of the cyanobacterium of the *A. filiculoides* symbiosis. In brief, proteomic analyses revealed that processes related to energy production, nitrogen, and carbon metabolism and stress-related functions (e.g., superoxide dismutase and peroxiredoxins) were upregulated in the cyanobiont compared with a free-living strain, whereas photosynthesis and metabolic turnover rates were downregulated (Ekman et al. 2008). Genome sequencing of the cyanobiont of *A. filiculoides* strongly suggests that these cyanobionts are at the initial phase of a transition from a free-living organism to a nitrogen-fixing plant entity. There has been coevolution between *Azolla* and the cyanobiont with genome degradation and signs of reductive genome evolution resulting in an organism devoted to nitrogen fixation and devoid of autonomous growth (Ran et al. 2010). Noteworthy is the loss of function within gene categories for basic metabolic processes such as glycolysis, replication, and nutrient uptake. This genomic analysis now opens the door for obtaining a much better understanding of this ecologically and evolutionarily important symbiosis.

The Cycad Symbioses

Introduction

First appearing in the Pennsylvanian era some 300 million years ago, cycads are the most primitive and longest-lived of present day seed plants (gymnosperms). They are also the only gymnosperms that enter into symbiotic associations with cyanobacteria. Once plentiful during the Jurassic, occupying far reaching habitats stretching from Alaska and Siberia to the Antarctic, they are now found in diminishing numbers in certain subtropical and tropical regions of mostly the southern hemisphere, including Australia, parts of Southern Asia, and South Africa (Brenner et al. 2003; Vessey et al. 2005). There are two or three extant families with some 300 species in 10 genera (Chaw et al. 2005; Vessey et al. 2005; Bergman et al. 2007; see also Lindblad 2009). Cycads are long-lived, cone-bearing evergreen palm-like plants which can reproduce either asexually (producing stem offshoots or suckers) or sexually. They grow terrestrially, with the exception of *Zamia pseudoparasitica* which is the only true epiphytic cycad and hangs from branches by the tap and lateral roots (Stevenson 1993).

In general, cycads have a stout trunk with a large crown of tough spiny leaves and can vary in height from a few tens of centimeters to almost 20 m at maturity. Most cycads produce different root types—a thick taproot that extends some 9–12 m beneath the soil surface, lateral roots, and coralloid roots which are highly specialized lateral roots named for their resemblance to coral. Coralloid roots exhibit negative geotropism, growing sideways and upward toward the soil surface, and are the sites in which symbiotic cyanobacteria can be found (Costa and Lindblad 2002; Lindblad and Costa 2002; Brenner et al. 2003; Vessey et al. 2005; Bergman et al. 2007, 2008).

Cyanobacteria living in association with cycads were first reported in the nineteenth century (Reinke 1872), and this partnership is still the only known example of a naturally occurring plant root-cyanobacterial symbiosis. All cycad species examined to date are able to form symbiosis with nitrogen-fixing cyanobacteria, visible as a dark blue-green band (the cyanobacterial zone) between the inner and outer coralloid root cortex (*Fig. 16.9*). The association between the cycad *Zamia furfuracea* and its native cyanobiont *Nostoc* sp. strain FUR 94201 has been separated and reconstituted successfully under axenic laboratory conditions (Ow et al. 1999). The same authors also showed for the first time that a cycad symbiosis could be established with the soil cyanobacterium *Nostoc* 2S9B, a strain previously shown to form loose associations with wheat roots. The ability of cycads to thrive in nutrient-poor soils is often attributed to the associations they form with cyanobacteria. Nitrogen fixation in cycads not only contributes to the nitrogen metabolism of the plant but also up to 18.8 kg N ha^{-1} $year^{-1}$ to the local nitrogen economy (see: Rai et al. 2000; Vessey et al. 2005).

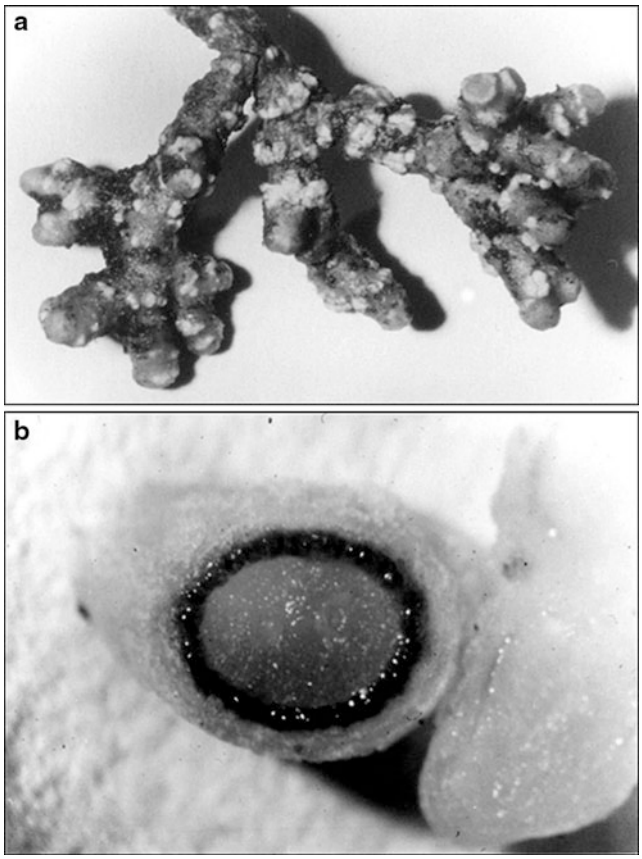


Fig. 16.9
The cycad-*Nostoc* symbiosis. (a) A cycad coralloid root, the site of cyanobacterial infection. (b) Transverse section of the root showing the dark cyanobacterial band between the inner and outer cortical layers [(a) From Lindblad et al. (1985a) with permission. (b) From Rai et al. (2000) with permission]

Heterotrophic bacteria have been found associated with cyanobacteria recovered from coralloid roots (Chang et al. 1988), although these bacteria have not been found in the cyanobacterial zone within the coralloid cortex (Grilli Caiola 1980). However, bacteria have been found inside the periderm of the coralloid roots of several different cycads (Joubert et al. 1989). It has been suggested that phenolic substances, detected in the mucilaginous material of the cyanobacterial zone and in the cortical cells surrounding the cyanobacterial zone, have antimicrobial properties that exclude organisms other than cyanobacteria (Grilli Caiola 1980; Obukowicz et al. 1981). Phenolics are among the most widespread plant secondary metabolites and have been shown to function as signaling molecules in the establishment of legume-rhizobial symbiosis and vesicular-arbuscular mycorrhiza, and there is some evidence that phenolic compounds may also influence the formation of symbiosis between cyanobacteria and cycads, as well as their metabolism (see Lobakova et al. 2004).

Cycads and Toxicity

The toxic properties of cycads have been noted for centuries, with the azoxyglycosides, cycasin and macrozamine, and the neurotoxic nonprotein amino acid β-methylamino-L-alanine (BMAA), receiving the most documentation. BMAA synthesis has been associated with the cyanobionts of cycads (Cox et al. 2003) as well as the host plant (e.g., Vega and Bell 1967; Polsky et al. 1972; Marler et al. 2010) and has since been found in all known groups of free-living cyanobacteria (Cox et al. 2005; Banack et al. 2007). BMAA has been linked to the high incidence of the progressive neurodegenerative disease amyotrophic lateral sclerosis/parkinsonism-dementia complex (ALS-PDC) in the Chamorro people on the island of Guam. They were thought to have acquired damaging levels of the toxin through the ingestion of cycad seed-eating flying foxes in which the toxin is believed to have been "biomagnified" (Banack and Cox 2003; Cox et al. 2003; Banack et al. 2006). However, this hypothesis is controversial (see Marler et al. 2010; Snyder and Marler 2011) not least because of difficulties in reliable separation and detection of BMAA. Some groups have confirmed the presence of BMAA in cyanobacteria and cycad seeds (Esterhuizen and Downing 2008; Spáčil et al. 2010), whereas contradictory results have been obtained following analysis of underivatized samples (e.g., Rosén and Hellenäs 2008; Krüger et al. 2010). These latter studies were, however, able to detect 2, 4-diaminobutyric acid (DAB), a neurotoxic isomer of BMAA (Rosén and Hellenäs 2008; Krüger et al. 2010). The recent work by Banack et al. (2010) addressed the issues concerning effective BMAA analysis, and they were able to reliably and consistently separate BMAA from 2,4 DAB as well as distinguish it from other compounds previously mistaken for BMAA during chloroformate derivitization for GC analysis (Banack et al. 2010). They concluded that cyanobacteria do indeed produce BMAA and its neurotoxic structural isomer 2, 4-DAB.

The biological significance of the toxins remains unclear, although possible roles include protection from herbivory, competition with other plants, or antibacterial and antifungal defense mechanisms (Castillo-Guevara and Rico-Gray 2003). BMAA is, however, heavily concentrated in the coralloid roots, raising the suggestion that the primary function of this toxin is not anti-herbivory (Marler et al. 2010). An alternative role might be in communication involved in the initiation and maintenance of the symbiotic association (Marler et al. 2010; Snyder and Marler 2011).

The Symbionts

The cyanobionts of cycads are filamentous, heterocystous species, largely restricted to the genus *Nostoc*, although *Calothrix* spp. have occasionally been found (Grobbelaar et al. 1987; Costa and Lindblad 2002; Rasmussen and Nilsson 2002; Gehringer et al. 2010; Thajuddin et al. 2010). Cycads can host multiple cyanobacterial strains in single plants as well as in single roots (Zheng et al. 2002; Thajuddin et al. 2010). However, taxonomic studies have revealed little if any specificity between cycads and their cyanobionts (Lindblad et al. 1989; Lotti et al. 1996; Costa et al. 1999; Zheng et al. 2002; Costa et al. 2004; Gehringer et al. 2010); the cyanobiont species found within a host plant is probably determined by the predominant symbiotically competent species available within the immediate rhizosphere (Gehringer et al. 2010).

Establishment of Symbiosis

Coralloid root development begins with the initiation of precoralloid root formation in the seedling (Rai et al. 2000; Lindblad 2009). Cyanobacteria are not found in precoralloids, and their presence is not necessary for the initiation of precoralloid development (Staff and Ahern 1993), although exposure to light is considered significant in many cycad genera (Webb 1983a, b). Infection by cyanobacteria can occur at any stage of precoralloid root maturation, and their presence triggers further growth and the developmental changes required to transform precoralloids into coralloids. The mode of entry of the cyanobacteria is still unclear, although suggested access points have included lenticels, breaks in the dermal tissue, or via the papillose sheath (see Bergman et al. 2007; Lindblad 2009). In addition, bacteria and fungi in the cycad rhizosphere may cause local degradation of the cell wall, enabling the cyanobacteria to penetrate the root (Lobakova et al. 2003). Following entry into the root, the cyanobiont migrates toward the cyanobacterial zone, between the inner and outer cortex, through a channel created through the outer cortex, believed to be caused by the separation, distortion, and destruction of cortical cells (Nathanielsz and Staff 1975).

The process of coralloid formation is irreversible, with one of the most significant changes being a conversion from negative to positive geotropism, resulting in growth sideways and upward toward the soil surface. Other changes include the loss of the papillose sheath, proliferation of apical lenticels, and early differentiation of the conspicuous cyanobacterial zone (Ahern and Staff 1994; see also Rai et al. 2000). Some cycad cells within the cyanobacterial zone undergo a distinct differentiation process to interconnect the two adjacent cortical layers, and this may facilitate the transfer of nutrients between the partners (Lindblad et al. 1985a; Lindblad et al. 1991; Pate et al. 1988; Costa and Lindblad 2002; Vessey et al. 2005; see also Lindblad 2009). Although the cyanobiont location is extracellular, there have been reports of intracellular cyanobionts in *Cycas revoluta* Thunb. and *Macrozamia communis* L. (see Lindblad 2009 and references therein).

Symbiotic Competence: Fit to Infect?

So, how do cyanobacteria living in the soil reach the sites of infection within the coralloid roots of cycads? As is the case with other plant hosts, cyanobacteria entering into functional symbiosis with cycads produce transient motile filaments

known as hormogonia that are highly adapted to sense and respond to environmental stimuli, including chemicals released by potential host plants. Water extracts from macerated seeds of the cycad *Zamia furfuracea* induce the development of motile hormogonia (Ow et al. 1999) and exhibit some chemoattractive properties. Chemotaxis of hormogonia in response to plant-derived attractants is likely to be of particular importance in the infection of cycad tissue, where the sites of infection receive little or no light, because the plant-derived signals must be able to override the natural phototactic response of the hormogonia.

Other Symbiotic Competence Factors

Laboratory attempts to reconstitute a functional symbiosis between *Nostoc* PCC 73102 (originally isolated from the cycad *Macrozamia* sp.) and *Nostoc* ATCC 29133 (believed to be the same isolate as PCC 73102 but with a different laboratory history and morphology; see Ow et al. 1999 and references there in) have been unsuccessful (Ow et al. 1999). This is surprising because, as described elsewhere in this chapter, *Nostoc* ATCC 29133 readily enters into functional symbiosis with a wide range of plants such as liverworts, hornworts, and the angiosperm *Gunnera*. This implies the existence of a recognition and compatibility selection process that is able to select certain cyanobacteria and exclude others. Alternatively, successful cyanobionts may have evolved mechanisms to protect against or disguise themselves from the host's natural defense system.

Carbon Metabolism

As the coralloid roots of most cycad species are located beneath the soil surface, their cyanobionts receive little or no light and are assumed to have a heterotrophic metabolism, possibly using fixed carbon supplied by the host and/or by their own dark CO_2 fixation (Lindblad 2009). Freshly isolated cyanobionts from *Cycas revoluta* coralloid roots fail to fix CO_2 in vivo under light or dark conditions (Lindblad et al. 1987), but their crude extracts have similar activities of RuBisCo and phosphoribulokinase to those in free-living cultures (Lindblad et al. 1987). The lack of CO_2 fixation by the cyanobiont might be due to a reversible inhibitor of RuBisCo, which is lost by dilution in the in vitro assay (see Meeks and Elhai 2002). Alternatively, the photosystems of the cyanobiont may be nonfunctional, or other enzymes of the Calvin cycle may be lacking (Lindblad et al. 1987).

In a study of the *Nostoc*-like cyanobiont of *Zamia skinneri*, a fully developed photosynthetic apparatus containing thylakoids with distinct phycobilisomes, harboring phycobiliproteins, was revealed (Lindblad et al. 1985a). Carboxysomes were also noted, although photosynthesis is unlikely to occur as the coralloid roots were collected from below the soil surface. However, high rates of photosynthetic oxygen evolution have been found in the cyanobacteria isolated from the roots of *Cycas circinalis* which are known to develop at or close to the soil surface where the cyanobacteria may be exposed to light (Perraju et al. 1986). Nevertheless, it is unclear why the dark-associated cycad cyanobionts retain a full photosynthetic apparatus, associated pigments, and carbon-fixing potential (cellular levels of RuBisCo are comparable with those in free-living counterparts).

Nitrogen Metabolism

Nitrogenase protein is restricted to the heterocysts, including contiguous heterocysts, in both free-living heterocystous cyanobacteria and those in symbiosis with cycads (Bergman et al. 1986). Nitrogenase activity is some three- to fivefold higher in cycad-associated *Nostoc* than in the free-living cultures (Lindblad et al. 1985b). Although nitrogenase activity parallels increasing heterocyst frequency, it reaches a maximum at heterocyst frequencies of around 25–35 % (Lindblad et al. 1985b) at a location where single heterocysts predominate, and declines thereafter, although heterocyst frequency continues to increase and contiguous heterocysts become common (Lindblad et al. 1985b). The possibility that some of the heterocysts within these clusters are metabolically inactive cannot be ruled out (Bergman et al. 1986). Indeed, the decrease in nitrogenase activity observed in older parts of the coralloid roots of *Cycas* and *Zamia* is believed to be in part due to the aging of the cyanobacteria located there (Lindblad 1990).

Cyanobacteria freshly isolated from older coralloid root sections, as well as those located close to the apex of *Macrozamia riedlei*, an Australian cycad in which the coralloid roots can develop up to 0.5 m below the soil, show marked light-stimulated nitrogenase activity (measured by both acetylene reduction and $^{15}N_2$ fixation), providing they are maintained under low (<1 %) O_2 levels (Lindblad et al. 1991). The low level of nitrogenase activity recorded in freshly isolated cyanobionts incubated in darkness probably results from loss or damage of heterotrophic mechanisms that previously functioned to provide the necessary ATP to support nitrogenase activity within the intact coralloid roots (Lindblad et al. 1991; see also Lindblad 2009). The authors suggested that damage caused by separation of the cyanobacteria from the host plant disrupts the intercellular microenvironment and any biochemical interactions provided by the intact cyanobiont-coralloid root association (Lindblad et al. 1991).

In free-living cyanobacteria, the ammonia derived from nitrogen fixation is primarily assimilated via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Muro-Pastor et al. 2005; Flores and Herrero 2005). In many cyanobacteria-plant symbioses, the release, to the host, of ammonia that would otherwise be assimilated by the cyanobiont is achieved partly by a host-mediated decrease in GS activity in the cyanobiont. However, xylem sap analysis of freshly detached *Nostoc*-colonized coralloid roots has revealed that the nitrogen fixed by the cyanobiont is instead translocated to the host as either a combination of the amino

acids citrulline and glutamine (in Zamiaceae) or glutamine alone (although in the Boweniaceae and Cycadaceae glutamic acid is also present; Pate et al. 1988; Costa and Lindblad 2002; Bergman et al. 2007). The possibility that ammonium is not the transferred N-solute in cycad associations is supported by the findings that cyanobionts of *Cycas revoluta*, *Ceratozamia mexicana*, and *Zamia skinneri* all have high in vitro GS activity and GS protein levels similar to those found in free-living cyanobacteria, including strains originally isolated from cycads (Lindblad and Bergman 1986).

Despite supplying the host with most of the nitrogen they fix, the cyanobionts do not show signs of nitrogen starvation, as they retain abundant sources of combined nitrogen. These include cyanophycin, which is a specialized nitrogen reserve (consisting of a copolymer of arginine and aspartic acid), carboxysomes, and phycobiliproteins (accessory photopigments), all of which can be degraded under conditions of nitrogen starvation (Meeks and Elhai 2002).

Other Adaptations to Life in Symbiosis

Cycad cyanobionts generally show little morphological change compared with their free-living counterparts, apart from an increased heterocyst frequency. Baulina and Lobakova (2003a, b) observed cyanobionts with vegetative cells and heterocysts showing considerable degradation of the peptidoglycan layer. However, it is not clear if such cells are functional or are in various states of senescence. In free-living cyanobacteria, heterocysts largely occur singly at almost regularly spaced intervals within a filament of photosynthetic vegetative cells (reviewed by Zhang et al. 2006). In return for the fixed carbon (possibly sucrose; see Meeks and Elhai 2002 for further discussion) they receive from neighboring vegetative cells, the heterocyst provides fixed nitrogen. In symbiosis, there is a developmental gradient of heterocysts from low (a heterocyst frequency of 16.7 % of total cells) in the growing tips of the coralloid roots to high (46 %) in the base (older parts) of the roots. Indeed, at the very growing tips of the coralloid roots of some cycad species are short "free-living" type filaments that resemble hormogonia. There are few heterocysts, those present being restricted mostly to the filament poles (Grilli Caiola 1980). In various cycad species, multiple contiguous heterocysts (double to quadruple) are found frequently in older root tissue but rarely at the tip (see Lindblad et al. 1985a, 1985b; Lindblad et al. 1991: also reviewed by Lindblad 2009). However, these contiguous heterocysts in older tissue may not be metabolically active (Bergman et al. 1986).

Cyanolichens

Introduction

Lichens are associations of symbiotic fungi and green algae (bipartite lichens) or symbiotic fungi, green algae, and cyanobacteria (tripartite lichens). A lichen thallus is quite distinct in appearance from either of its symbionts, and its name refers to the dominating fungal partner (the mycobiont). Lichen thalli represent an integration of the mycobiont's heterotrophic metabolism and the autotrophic metabolism of the photosynthetic partners (the photobionts: green algae and cyanobacteria). In tripartite lichens, the cyanobacterial partner (the cyanobiont) is also referred to as the "secondary photobiont," whereas the green algal partner is referred to as the "primary photobiont." All lichens having a cyanobiont, either as the sole photobiont or as a secondary photobiont, are called "cyanolichens." For lack of space, this review on cyanolichens is brief. The reader can find further details in books and reviews elsewhere (Galun 1988; Ahmadjian 1993; Nash 1996; Rai et al. 2002; Rikkinen 2002). The journal Bryologist regularly lists recent literature on lichens, and a literature search is also possible at Mattick's Literature Index website ([{http://www.toyen.uio.no/botanisk/bot-mus/lav/sok_rll.htm}]).

There are approximately 1,550 known species of cyanolichens, representing roughly 12–13 % of all known lichens; among these, two-thirds are bipartite and the rest tripartite species. Lichen symbioses are thought to have arisen independently on several occasions. An estimated 100 lichenization events have occurred during diversification of extant fungi (Aptroot 1998; see also Rikkinen 2002).

The Symbionts

Mycobionts

The current classification of fungi is in transition, and molecular approaches are being used to fine-tune it (Tehler et al. 2000; see also Rikkinen 2002). Approximately 13,500 species of lichen-forming fungi presently belong mostly to the Ascomycetes (98 %) and very few to the Basidiomycetes (1.6 %) and fungi imperfecti (0.4 %). About 15–18 orders of Ascomycetes (nearly 130 genera from 50 families) include lichen-forming taxa (see Rikkinen 2002). Most are from two orders, the Lecanorales and Lichinales. Nearly 1,700 species of fungi associate with different types of cyanobacteria. A fairly comprehensive list of these has been provided earlier (Rikkinen 2002).

Cyanobionts

A variety of heterocyst-producing and unicellular cyanobacteria occur as cyanobionts in cyanolichens where the mycobiont is an ascomycete. Among heterocystous forms, *Nostoc* is the most common. Others are *Scytonema*, *Calothrix*, *Dichothrix*, and *Fischerella* (including *Hypomorpha*, *Stigonema*, and *Mastigocladus*). Unicellular forms that occur as cyanobionts in cyanolichens include *Gloeocapsa* (also *Chroococcus*), *Gloeothece*, *Synechocystis* (also *Aphanocapsa*), *Chroococcidiopsis*, *Hyella*, and *Myxosarcina* (see Rai et al. 2000; Rikkinen 2002). The range of cyanobionts in cyanolichens where the mycobiont is

a basidiomycete is rather limited. Only two cyanobacteria (*Chroococcus* and *Scytonema*) are reported as cyanobionts in basidiolichens (see Schenk 1992).

Analyses of $tRNA^{Leu}$ (UAA) introns and 16S rDNA sequences have been used as genetic markers to study the diversity of *Nostoc* cyanobionts (Paulsrud and Lindblad 1998; Paulsrud et al. 1998, 2000, 2001; Lohtander et al. 2002; Rikkinen et al. 2002). These studies have shown that genetic variation among lichen-forming *Nostoc* strains is considerable. Within symbiotic *Nostoc* strains, there seem to be several subgroups. For example, one subgroup of *Nostoc* strains seems to occur only in epiphytic cyanolichens, whereas another includes strains that occur as cyanobionts in terricolous cyanolichens and other symbiotic systems (Rikkinen 2002; Rikkinen et al. 2002). Miura and Yokoto (2006) have reported the occurrence of two cyanobionts in the same lichen. Based on morphological observations and 16 s rDNA sequences of cyanobacterial isolates from lichens, they reported the occurrence of *Nostoc*, *Calothrix*, *Cylindrospermum*, *Phormidium*, *Leptolyngbya*, *Microcystis*, and *Chroococcidiopsis*.

The Lichen Thallus

Lichen thalli have a stable and organized structure quite distinct from any of their symbionts. The thalli appear to be crustose (small lobes and scales; e.g., *Collema*), foliose (flat and dorsiventral lobes; e.g., *Peltigera*), or fruiticose (round or flat thalli, upright, or hanging down from the substratum; e.g., *Stereocaulon*). In foliose or fruticose thalli, the fungal hyphae form an outer pseudoparenchymatous zone (the cortex) that covers or encloses a more loosely interwoven medulla. Within the thallus, the partners remain extracellular to each other and can be isolated and grown in culture, but the symbiosis is fairly stable in nature because of the balanced and synchronized growth and development of the symbionts. Thinner cell walls (less sheath material) and specialized hyphae and haustoria, showing transfer cell ultrastructure, enable close contact between the mycobiont and the cyanobiont. Since the bulk of the thallus consists of the heterotrophic mycobiont, the thallus interior is microaerobic (see Rai et al. 2000).

In bipartite lichens, cyanobionts either are dispersed throughout the thallus (e.g., *Collema*) or occupy a distinct layer below the upper cortex (e.g., *P. canina*). In tripartite lichens, the cyanobiont is located in cephalodia, which occur at the upper surface of the thallus (external cephalodia; e.g., in *P. aphthosa*) or inside the medulla (internal cephalodia; e.g., in *Nephroma arcticum*). In some cases, internal cephalodia are found close to the lower surface of the thallus (e.g., in *P. venosa*). In tripartite lichens, direct contact between the cyanobiont and the phycobiont (green algal partner) is never direct.

Lichen symbioses perpetuate by direct transmission of the cyanobiont from one generation to the next and, as a result of the acquisition, by the mycobiont of fresh cyanobiont from the environment. For example, a lichen thallus can develop from propagules (phyllidia, isidia, soredia, and hormocystangia) of a preexisting thallus (direct transmission) or from fresh synthesis (fresh acquisition of cyanobiont from the environment). The former mode of transmission allows prolonged continuity of the partners. Similar modes of cyanobiont acquisition also apply to the development of cephalodia (see Rai et al. 2000). Cyanobionts are essential for the formation of thalli or cephalodia in cyanolichens. They may stimulate thallus morphogenesis but do not determine the kind of thallus formed; the mycobiont determines the structure and chemistry of a cyanolichen. Different lichen fungi form different lichen thalli even if associating with the same cyanobiont (see Rai 1990; Rai et al. 2000, 2002).

Because they are slow growing, the initiation and development of lichens is difficult to study in nature. Development of a lichen thallus afresh involves germination of the mycobiont spore, development of the hyphal mat, contact, recognition, and acquisition of the cyanobiont, and structural-functional integration of the symbionts. While a thallus may result within months when starting from propagules, it takes years when starting from isolated partners. During laboratory synthesis of lichens, the partners initially form undifferentiated aggregates that later differentiate into thalli (see Rai et al. 2000). Fresh synthesis in nature may also start from mycobiont hyphae that become detached and acquire a fresh cyanobiont (Smith and Douglas 1987).

Development of each cephalodium is a new event. External cephalodia develop on the main thallus by entrapment of a cyanobiont by hairs on the thallus surface, followed by involvement of medullary hyphae immediately below. Internal cephalodia may develop in a similar fashion starting with cyanobiont entrapment by cortical hyphae or rhizines. The cyanobiont, enmeshed by a thick layer of mycobiont, is pressed into the thallus where the cephalodium eventually develops. New cephalodia may develop from hormogonia released by earlier cephalodia (Stocker-Wörgötter 1995), ensuring cyanobiont homogeneity among cephalodia of a thallus. In laboratory synthesis, however, cephalodia developed by attachment of hyphae from primordia (containing cyanobiont and mycobiont) to the green thallus (Stocker-Wörgötter and Turk 1994). The latter mode of cephalodia development, if prevalent in nature, should cause considerable heterogeneity among symbiont populations within a single thallus, but this is not the case. Occasional reports of different cyanobionts (see Rai 1990) or different strains of a cyanobiont (Paulsrud et al. 2000) among cephalodia of a single thallus may, however, indicate instances of cephalodia development by capture of a fresh cyanobiont in some lichens. Entry of the cyanobiont for development of internal cephalodia is from the lower surface of the thallus, but occasionally, when the cyanobiont enters from above, the phycobiont layer is pressed deep into the medulla.

Recognition and Signal Exchange Between Partners

For the right symbionts to enter into a lichen symbiosis, signal exchange must occur between the partners. Transformation of

Nostoc colonies into the symbiotic state occurred without the necessity for direct contact with the mycobiont during resynthesis of *Peltigera praetextata* (Yoshimura and Yamamoto 1991). This suggests that the substance responsible for *Nostoc* transformation may be a diffusible soluble substance from the mycobiont. The exact identity of such a substance is not known, but lichen-forming fungi do produce a large number of unique secondary metabolites and compounds, and their possible roles in signal exchange need to be investigated. Lectins (glycoproteins) of mycobiont origin have been implicated in the recognition of the cyanobiont by a mycobiont (Rai 1990; Kardish et al. 1991; Lehr et al. 1995, 2000). Cyanobiont cell surfaces possess specific sugars, fimbriae (pili), and in some cases, lectins, which may have a role in recognition and adherence (Stewart et al. 1983; Kardish et al. 1991; see Rai et al. 2000).

Direct observations, lectin-binding experiments, and $tRNA^{Leu}$ intron analysis all indicate a broader cyanobiont-mycobiont specificity in lichens than that in other cyanobacterial symbioses. Different lichen species can have the same cyanobiont, and different cyanobionts have been reported among cephalodia of a single lichen thallus. Different *Nostoc* strains have been found in different lichen species from the same site, while different lichen species from distant places had the same *Nostoc* strain. In chimeroid thalli, both bipartite and tripartite morphotypes are reported to have the same cyanobiont strain (Paulsrud et al. 1998, 2000, 2001). Overall, there is a great deal of cyanobiont diversity among the lichens, and much of it might be contributed by the mode of cyanobiont acquisition during the development of the lichen thallus and cephalodia (Rai et al. 2000).

Many cyanolichens share similar environmental requirements and may depend on a common pool of cyanobionts. Many cyanolichen species having identical cyanobiont strains co-occur in a particular habitat, forming characteristic communities or "guilds" (Rikkinen et al. 2002). Within a guild, the cyanobionts of all lichens are closely related, but the mycobionts are not. While some guilds include different mycobiont genera or even families, some closely related mycobionts belong to different guilds (associate with different types of cyanobionts).

Structural-Functional Changes

Cyanobionts undergo structural-functional changes in the symbiosis that permit a close interaction and development of nutrient exchange between the partners. These changes include increased cell size, altered cell shape, lack of polyphosphate reserves, fewer carboxysomes, less sheath material, and slower growth and cell division (Rai et al. 2000).

The cyanobionts are photosynthetically active and fix CO_2 via the C_3 pathway. In addition, there is a significant level of dark CO_2 fixation (15–20 % of that in the light) via the C_4 pathway (Rai et al. 2000; Palmqvist 2002). However, CO_2 fixation by cyanobionts in internal cephalodia, particularly those on the lower surface (e.g., *Peltigera venosa*), may be minimal due to low light and RuBisCo. In the tripartite *Nephroma arcticum*, the *Nostoc* cyanobiont has 70 % fewer carboxysomes compared with that in the bipartite *P. canina* (Bergman and Rai 1989).

In free-living cyanobacteria, heterocysts are regularly spaced and represent about 5–10 % of the cell population. There is a change in the spacing pattern of heterocysts and an increase in their frequency in the cyanobionts in tripartite lichens (heterocyst frequency 15–35 %) but not in bipartite lichens. Heterocyst frequency correlates with the status of fixed carbon in the cyanobiont; in bipartite lichens, the cyanobiont bears the burden of providing both fixed nitrogen and fixed carbon to the mycobiont, whereas in tripartite lichens, it provides fixed nitrogen only. Indeed heterocyst frequency increases when *Nostoc* isolates are grown in the dark with sugars. In many cyanobacterial-plant symbioses, where the cyanobiont receives fixed carbon from the plant host, heterocyst frequencies of up to 80 % can occur (see Rai et al. 2002).

In free-living cyanobacteria, glutamine synthetase (GS) is the primary ammonia assimilating enzyme, and GS levels in heterocysts are twofold higher than those in vegetative cells (Bergman et al. 1985). In cyanobionts, the GS activity and protein levels decrease by over 90 %, and the remaining GS is uniformly distributed among heterocysts and vegetative cells (Bergman and Rai 1989; Rai 2002). GS activity is undetectable in the mycobiont, but mycobiont hyphae in contact with cyanobiont cells show high levels of nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent glutamate dehydrogenase (GDH) activity.

Nitrogen fixation occurs in all lichens containing heterocystous cyanobionts. The rates are higher in tripartite lichens owing to the higher heterocyst frequency of the cyanobiont (see Rai et al. 2000; Rai 2002). In contrast to free-living forms, cyanobionts in bipartite lichens and in excised cephalodia continue to fix N_2 even in the presence of nitrate or ammonia (Stewart and Rowell 1977; Rai et al. 1980). However, nitrogen fixation by the cyanobiont in cephalodia attached to the main thallus of the tripartite lichen *P. aphthosa* was repressed by nitrate and ammonia. The effect was obviously mediated via the phycobiont. Significant levels of N_2 fixation have also been reported in darkness, and under these conditions ammonia has an inhibitory effect (Rai et al. 1981a, 1983b). As in the free-living forms, nitrogenase is located only in the heterocysts, despite the microaerobic conditions in lichen thalli (Bergman et al. 1986).

The extent of the changes described above varies from young to older, more mature parts of the thallus. While growth rate gradually declines, cell division and GS levels, the levels of N_2 fixation, CO_2 fixation, and heterocyst frequency of the cyanobiont increase. There is a parallel increase in the GDH activity in the mycobiont (Rowell et al. 1985; Hill 1989; Rai 2002). Still undetermined is whether these changes are caused by the mycobiont or by endogenous regulation due to special environmental conditions offered by the host in the symbiosis.

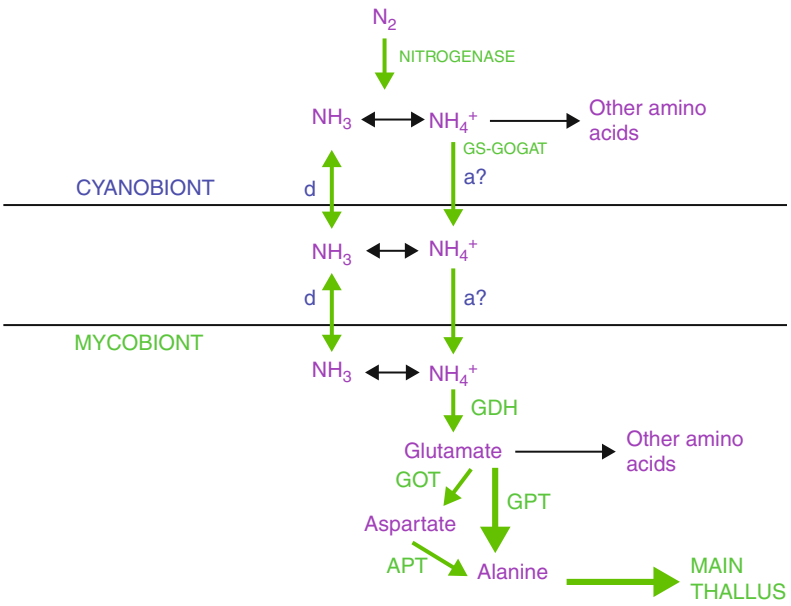


Fig. 16.10
Pathways of N-metabolism in *P. aphthosa*. a, active transport; d, diffusion. *GS* glutamine synthetase, *GOGAT* glutamate synthase, *GOT* glutamate-oxaloacetate transaminase, *GPT* glutamate-pyruvate transaminase, *GDH* glutamate dehydrogenase, *APT* aspartate-pyruvate transaminase

Nutrient Exchange

Most studies on nutrient exchange relate to carbon and nitrogen transfer from the cyanobiont to the mycobiont in foliose lichens, particularly *Peltigera* species. Such nutrient transfer is biotrophic in nature and varies along the lichen thallus. From young to mature parts of the lichen thallus, the cyanobiont increases fixation and release of nitrogen and carbon. Specialized mycobiont hyphae and haustoria showing transfer cell ultrastructure (TCU) may play an important role in the nutrient exchange (see Rai et al. 2000).

In bipartite cyanolichens, 70–80 % of the CO_2 fixed is released by the cyanobiont to the mycobiont. The transfer of fixed carbon occurs mostly in the light and in the form of glucose. Cyanobionts in tripartite lichens transfer little (<5 % of CO_2 fixed) or no fixed C to the mycobiont. Their primary role seems to be the provision of fixed nitrogen. It would be interesting to know whether the cyanobionts in internal cephalodia occurring deep in the medulla or on the undersurface of a lichen thallus actually receive any fixed carbon from the phycobiont (either directly or via the mycobiont). The glucose transferred to the mycobiont is converted to mannitol, which serves as both a C source and a physiological buffer. Mannitol production by lichenized fungi could be an effective way of sequestering the fixed carbon since the other partners cannot use it. The mechanism underlying glucose transfer is not fully understood, but the glucose is thought to originate from a glucan pool rather than directly from CO_2 fixation. Altered cell wall synthesis may lead to a diversion of sugars from cell wall synthesis to simple release. Release of glucose declines sharply and stops soon after the isolation of the cyanobiont, indicating the influence of mycobiont and symbiotic conditions in the thallus on this process (Smith and Douglas 1987; Meindl and Loos 1990; Rai 1990; Palmqvist 2002).

^{15}N tracer studies in *P. aphthosa* (tripartite) and *P. canina* (bipartite) have concluded that fixed N is transferred from cyanobiont to the mycobiont as ammonia (Rai et al. 1981b, 1983a). Over 90 % of the N_2 fixed in *P. aphthosa* (and about 50 % in *P. canina*) is released by the cyanobiont because GS in heterocysts is repressed. The partitioning of fixed N among the partners is proportionate to their contribution to the thallus composition. In *P. aphthosa*, the ammonium released by the cyanobiont is primarily assimilated by the mycobiont in cephalodia, and the phycobiont receives fixed N via the mycobiont. The mechanism of ammonia release by the cyanobiont and its uptake by the mycobiont at the cyanobiont-mycobiont interface have not been investigated. However, diffusion of NH_3 from heterocysts can occur in the absence of ammonia assimilation by GS. Ammonia assimilation in the mycobiont occurs via GDH followed by aminotransferases. In pulse-chase experiments, much of the ^{15}N label accumulated as alanine in the mycobiont of *P. aphthosa* cephalodia. Alanine could be the principal compound transferred to the rest of thallus (*Fig. 16.10*).

Ecological Significance

Lichens are ubiquitous, occurring in terrestrial as well as aquatic habitats from the equator to the highest latitudes, at sea level to

9,000-m altitude, and in the wettest to driest habitats. They are excellent colonizers of nutrient-poor habitats (sand dunes, rocks, forest floors, and the surfaces of other vegetation), form dominant vegetation in tundra and arctic-alpine regions, and contribute significantly to the N economy of these ecosystems. Lichens are good bioindicators of air pollution.

Cyanobionts endow mycobionts with N and C autotrophy and thereby widen their potential habitats. In a lichen thallus, cyanobionts gain a safe habitat and protection from uncertainty of fluctuating nutrient availability and climatic conditions in nature.

Outlook

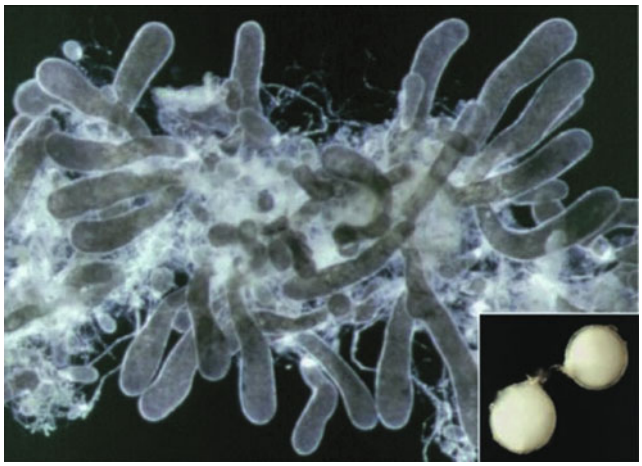
Many interesting aspects of the lichen symbioses remain to be elucidated. These include release and uptake of nutrients at the cyanobiont-mycobiont interface, cyanobiont acquisition, and regulatory mechanisms enforcing synchronized growth and development of the partners. Furthermore, whether the structural-functional changes in symbionts are a result of endogenous regulation due to the symbiotic environment (e.g., microaerobiosis, restricted growth, and cell division) or whether they are directly caused by the mycobiont will need to be resolved.

The *Geosiphon pyriformis* - *Nostoc* Endocyanosis and Its Relationship to the Arbuscular Mycorrhiza (AM)

The *Geosiphon pyriformis* Symbiosis

The fungus *Geosiphon pyriformis* (Kütz.) v. Wettstein (von Wettstein 1915) forms the only known fungal endocyanosis (endocytobiotic association with cyanobacteria). The coenocytic fungus forms unicellular, multinucleated cells ("bladders") of up to 2 mm in size (◉ *Fig. 16.11*), harboring endosymbiotic, filamentous cyanobacteria of the genus *Nostoc*. There have been only six reports describing this symbiosis in nature at locations ranging from eastern Germany to Austria. Probably, the symbiosis is geographically widespread in Central Europe but, due to its small size, rarely reported. Presently, field sites around the small village of Bieber in the Spessart Mountains (Germany) are the only known stable natural habitats worldwide (Mollenhauer 1992; Schüßler and Wolf 2005).

The species name "*Geosiphon pyriforme*" was sometimes used for the fungus as well as for the symbiosis because the latter was often regarded as a "phycomycetous lichen." Nowadays endosymbiotic associations are usually excluded from lichen definitions (Hawksworth and Honegger 1994). Thus, the species name should be used for the fungus only, also because phylogenetically the *Geosiphon* fungus belongs to the arbuscular mycorrhiza (AM)-forming and related fungi, the *Glomeromycota* (◉ *Fig. 16.12*). Here, the association between the fungus and cyanobacteria is referred to as the *Geosiphon-Nostoc* symbiosis, or simply the *Geosiphon* symbiosis, and the species name of the fungus is used in its orthographically correct form, *Geosiphon pyriformis* (Schüßler 2002).



◉ **Fig. 16.11**
The *Geosiphon-Nostoc* symbiosis, isolated from a laboratory culture on natural substrate, incubated in liquid medium. The *dark* bladders are about 1.5 mm in length. The *insert* shows *Geosiphon pyriformis* spores, which have a diameter of about 250 µm

The Symbionts

Geosiphon pyriformis

After its original description as *Botrydium pyriforme*, a siphonal alga (Kützing 1849), *Geosiphon pyriformis* (as *G. pyriforme*) was recognized as a phycomycete (fungus with aseptate hyphae; Knapp 1933). Sixty years later, based on suggestions by Walter Gams, it was suggested that *Geosiphon* could be related to *Glomus*-like fungi (Mollenhauer 1992). Such fungi form the arbuscular mycorrhiza (AM) symbiosis with land plants which is extremely important ecologically and economically, therefore verification of the phylogenetic relationship of *Geosiphon* would make it conceivable that *Geosiphon* may also be capable of such association.

Because the systematics of AM fungi in the last century was based mainly on the characteristics of their spore structure, morphological and ultrastructural criteria of *Geosiphon* spores were compared with those of some AM fungi (Schüßler et al. 1994). This indeed revealed similarities between *G. pyriformis* and AM fungi like *Diversispora epigaea* BEG47 (at that time named *Glomus versiforme*; see Schüßler et al. 2011). Final evidence showing that *Geosiphon* is closely related to AM fungi was based on small subunit ribosomal RNA (SSU rRNA) gene sequences (Gehrig et al. 1996). The AM fungi, together with *Geosiphon*, formed a distinct clade not closely related to any other group of the zygomycetes. Further sequence analyses (Schüßler 1999; Redecker et al. 2000b) showed that *Geosiphon* is closely related to an AM fungus forming two different spore morphs, at that time named *Acaulospora gerdemannii*.

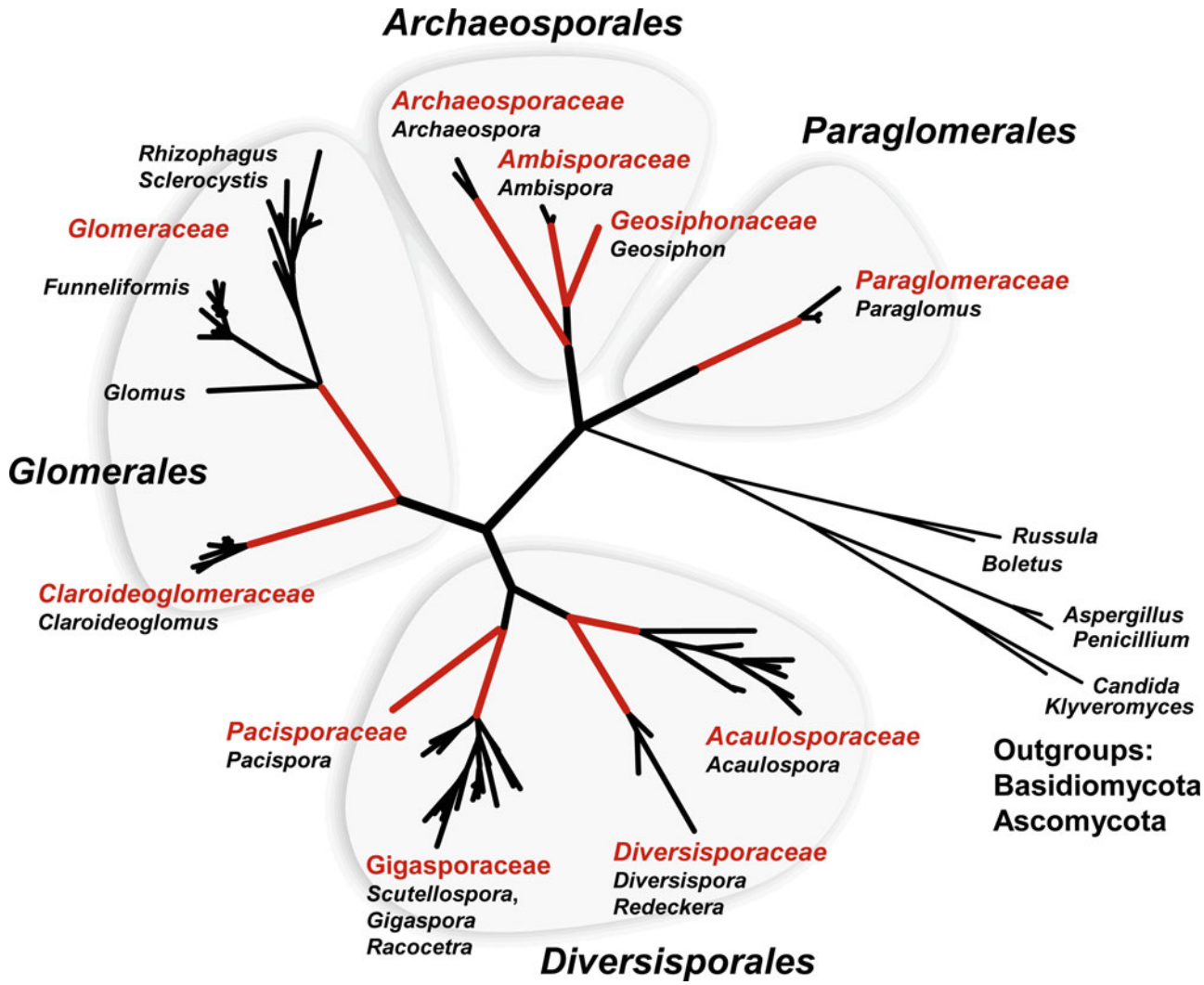


Fig. 16.12
Phylogenetic tree of AM fungi (*Glomeromycota*), including *Geosiphon* (Modified and updated from Schüßler et al. 2001; Schüßler and Walker 2010, http://www.amf-phylogeny.com). Families (represented by *red* branches) and genera are given, except for *Entrophospora* and *Otospora*, for which placement is yet unclear

Nowadays, the clade containing these lineages is defined as the order *Archaeosporales*, which represents one of the basal main phylogenetic lineages in the phylum containing the AM fungi and *Geosiphon*, the *Glomeromycota* (Schüßler et al. 2001). In this order, the *Geosiphonaceae* clusters as sister to the *Ambisporaceae*, thus appearing to be more derived than the *Archaeosporaceae*, which branch earlier. This means that *Geosiphon* does not represent a sister lineage to the AM fungi, as was sometimes wrongly suggested. It was the analysis of the phylogeny of *Geosiphon* that eventually led to the erection of the *Glomeromycota*, a widely accepted fungal phylum and, eventually to the phylogenetically based, revised classification of the *Glomeromycota* (Schüßler and Walker 2010).

The *Geosiphon-Nostoc* symbiosis attracted interest from the field of AM research. The AM symbiosis is formed by ~80 % of all vascular plants studied (Brundrett 2009) and moreover also by lower plants (Read et al. 2000; Schüßler 2000), despite their lack of roots. Considering this huge number of plants that form AM, it is obvious that the AM must be one of the most important factors in land ecosystems (Smith and Read 2008).

Nostoc punctiforme

The endosymbiont in the *Geosiphon* symbiosis (*Fig. 16.13*) is *N. punctiforme*, which belongs to a clade of cyanobacteria containing many symbiosis-forming members. In laboratory cultures (Schüßler and Wolf 2005), a strain that originally was isolated from the *Geosiphon* symbiosis was used (Mollenhauer

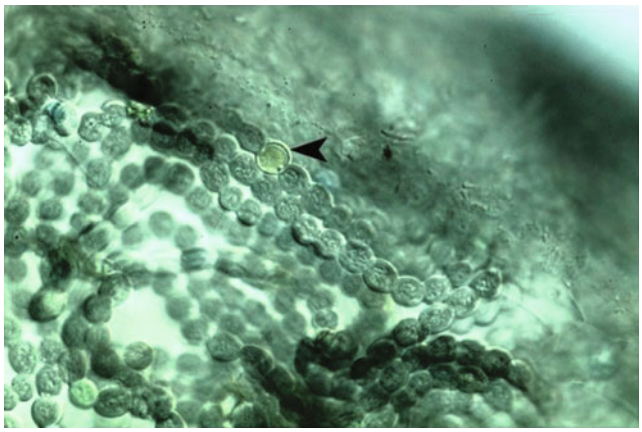


Fig. 16.13
Endosymbiotic *Nostoc* cells (about 7 × 6 μm in size), within a *Geosiphon* bladder. One heterocyst is in focus (*arrowhead*)

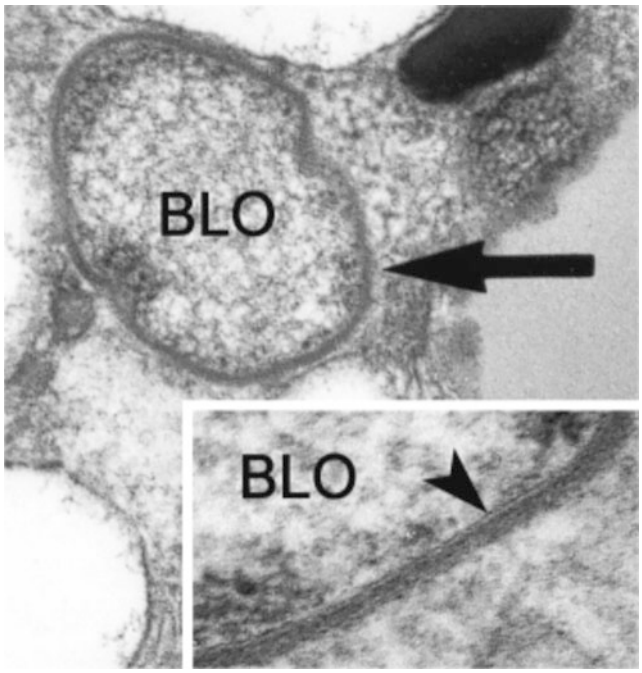


Fig. 16.14
Electron micrograph of a "bacteria like organism" (BLO) in *Geosiphon pyriformis*. BLOs have a diameter of about 0.5 μm and are not enclosed by a host membrane (*arrow*). The *insert* shows the plasma membrane of the BLO (*arrowhead*), as well as the thick murein sacculus. Recent studies show them to be *Mycoplasma*-related, despite the Gram-positive appearance

1992). However, various other strains of *N. punctiforme* from other symbiotic systems (e.g., *Anthoceros*, *Blasia*, *Gunnera*) are also capable of forming symbiosis with *G. pyriformis*. In the field, *G. pyriformis* was usually found together with *Anthoceros*, and the cyanobionts of *G. pyriformis* associate in symbioses with *Anthoceros* and *Blasia* (Mollenhauer 1992).

It has to be noted that *Geosiphon* harbors another prokaryotic endosymbiont, the so-called BLOs (bacteria-like organisms; *Fig. 16.14*), which are not enclosed by a fungal host membrane but live freely in the cytoplasm (Schüßler et al. 1996; Schüßler 2012). These endosymbiotic bacteria, and those living in most of the AM fungi that were studied for their occurrence, have the same typical ultrastructure. Because they are found in very diverse branches of the *Glomeromycota*, they were considered to be widespread, Gram-positive glomeromycotan symbionts (Schüßler et al. 1994).

The BLOs are indeed ancestral and typical endobacteria in AM fungi. New findings regarding their phylogeny and occurrence in very diverse AM fungal lineages (Naumann et al. 2010) showed that the BLOs are related to the cell-wall-lacking *Mollicutes*. We now know that they are monophyletic and laterally transferred within the AM fungi for more than 450 million years. Their phylogeny and biotrophic lifestyle are shared with the related mycoplasmas, despite the obvious difference of possessing a murein sacculus.

The *Geosiphon* symbiosis is facultative for one of the partners (*Nostoc* can be cultivated without the fungus) and obligate for the other one (*Geosiphon* is obligatory symbiotic). It is conceivable that the fungus is not restricted to the cyanobacteria as symbiotic partner but also forms symbioses with land plants (see below). However, this assumption is still speculative. Regardless, *Geosiphon* belongs to the *Glomeromycota*, and the *Nostoc* symbiosis bears functional and structural similarities to the AM. Thus, the *Geosiphon-Nostoc* symbiosis can play a role as a model symbiosis (Schüßler 2012) for the AM, which is difficult to investigate but extremely important. For example, the characterization of symbiosis-related genes is facilitated by use of this symbiosis (e.g., Schüßler et al. 2006).

Infection Process, Development, and Structure of the Symbiosis

Infection Process

Both symbiosis partners live in the upper layer and on the surface of humid soil, where they make contact. The interaction is considered to be specific for two reasons: (1) Only certain *Nostoc punctiforme* strains can form this symbiosis. (2) For a successful interaction with the fungus, *Nostoc* has to be differentiated into a specific stage represented by an early immobile stage of the cyanobacterial developmental cycle, the so-called primordium (Mollenhauer et al. 1996). The motile filaments (hormogonia) and late primordial, as well as vegetative stages of *Nostoc*, are not recognized by the fungus. When contacting *Nostoc*, the tip of the fungal hypha bulges out and surrounds part of a cyanobacterial filament, thus incorporating the *Nostoc* cells (*Fig. 16.15*). Usually, 5–15 *Nostoc* cells are taken up during this process, whereas the heterocysts are never incorporated but "cut off" by the fungus (see below). These events are documented in a scientific film available in German and English (Mollenhauer and Mollenhauer 1997).

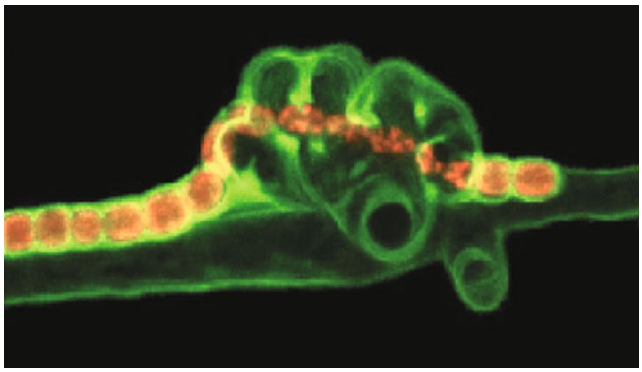


Fig. 16.15
Confocal laser scanning microscopy (CLSM) projection of a short hypha branching from a main hypha (horizontally oriented, 4–6 μm in diameter) and "bulging out" to enclose a part of a *Nostoc* filament. The extracellular polysaccharides of *Nostoc* and the outer layer of the fungal cell wall are labeled by the fluorescence-coupled lectin ConA (*green*). The *Nostoc* cells (*red* autofluorescence, ~4 × 3 μm in size) that are taken up by the fungal structure show strong deformations and irregular and reduced pigment fluorescence

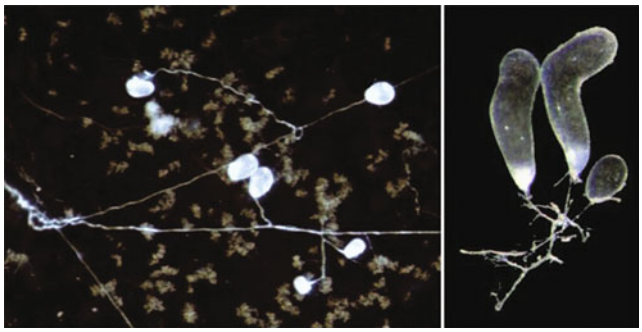


Fig. 16.16
Young *Geosiphon* bladders, 100–150 μm in size, formed on the fungal mycelium 7–10 days after initial uptake of the cyanobacteria (*left*). The irregular structures in the background are vegetatively growing *Nostoc* colonies. At the *right*, two mature bladders of about 1 mm length, together with a young bladder, are shown

Development of the Symbiosis

Studies on the development of the *Geosiphon-Nostoc* symbiosis showed that a successful interaction depends on the appropriate developmental stage of the cyanobacterium (Mollenhauer et al. 1996; Wolf and Schüßler 2005). The life cycle of *Nostoc* starts from akinetes (spore-like resting stages) leading to vegetative colonies. These colonies release motile trichomes (hormogonia) which are positively phototactic in dim light and negatively in strong light. As a consequence, the hormogonia often congregate just below the soil surface where they spread and meet their symbiotic partners. They eventually undergo a transformation into an aseriate stage called a primordium. This then differentiates into so-called vegetative cells, which divide and form gelatinous colonies ("thalli"). Only the very early primordial stage of *Nostoc* can interact with the fungal partner to give rise to the symbiotic consortium.

The life cycle of the fungal partner starts from resting spores formed in the upper soil layer. The spores (Schüßler et al. 1994) germinate by the outgrowth of a hypha (sometimes more than one), which branches to form a small mycelium of up to 2–3 cm in the soil. When a hyphal tip contacts a compatible early *Nostoc* primordium, the fungal hypha bulges out just below the apex. This bulging process is repeated several times so that eventually the hyphal tip forms an irregularly shaped structure surrounding a part of a *Nostoc* primordium. After this incorporation into the fungal hypha, large amounts of cytoplasm stream into this *Nostoc*-containing structure, which then starts swelling and develops the fungal bladder (*Fig. 16.16*).

Each individual incorporation event results in the formation of a single pear-shaped aboveground bladder (Knapp 1933). Each bladder represents a polyenergid cell, coenocytic with the fungal mycelium, in which the symbiotic *Nostoc* cells divide and become physiologically active. Laboratory culturing experiments have shown that, as for AM, phosphate limitation (1–2 μM) of the nutrient solution triggers the stable establishment of the symbiosis. N limitation seems not to be a crucial factor. The same situation is found in the natural habitat, so P limitation seems to be a driving factor for the establishment of this symbiosis.

Within the first hours after incorporation into the fungal cytoplasm, the *Nostoc* filaments become heavily deformed, and some cells may die during this process. The photosynthetic pigments degrade considerably (*Fig. 16.15*; Mollenhauer et al. 1996; Schüßler and Wolf 2005). These alterations and significant changes in ultrastructure suggest that during the initial state of endocytotic life, the incorporated cyanobacteria suffer severe stress. Within 2–3 days, the enclosed *Nostoc* cells recover and begin to multiply and grow to reach as much as six times the volume of free-living cells (Schüßler et al. 1996; Mollenhauer and Mollenhauer 1997). Under phosphate limitation, the endosymbiotic cyanobacteria divide much faster and form a much higher biomass compared with the free-living ones (unpublished). In the symbiosis, the *Nostoc* cells arrange in filaments in which heterocysts are formed with the same frequency as in the filaments outside the bladders (if cultured under nitrogen limitation). Mature *Geosiphon* bladders can then reach more than 2 mm in length and up to 6 months in lab cultures. They possess a turgor pressure of about 0.6 MPa (=6 bar) (Schüßler et al. 1995).

Structure and Compartmentation of the *Geosiphon* Bladder

The *Geosiphon* bladder is effectively a multikaryotic cell, coenocytic with the fungal mycelium in the soil. It shows a strong

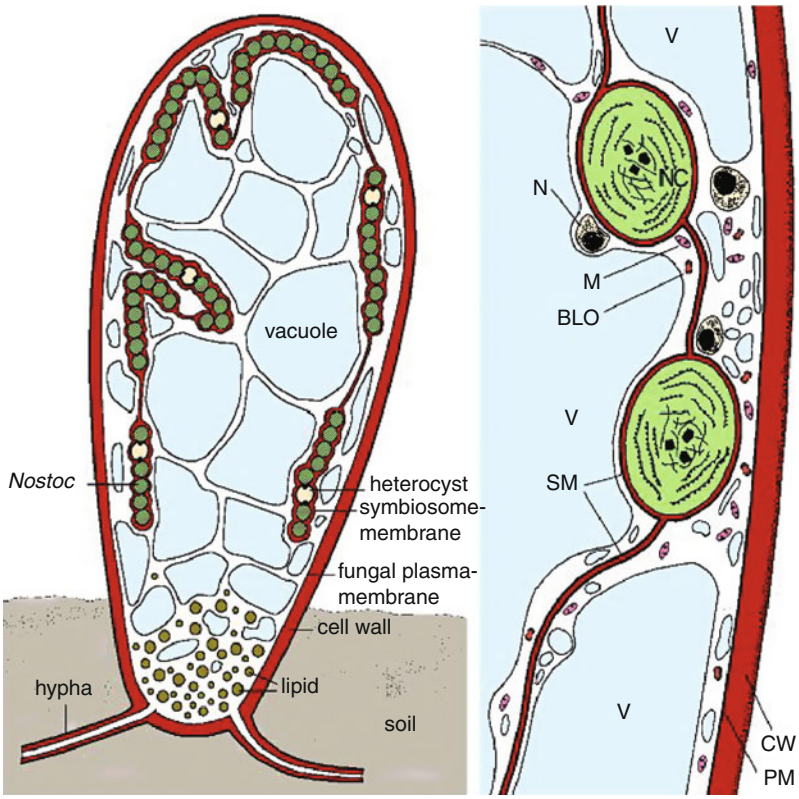


Fig. 16.17

Schematic representation of the compartmentation of the *Geosiphon-Nostoc* symbiosis (*left*). At the *right*, a magnification of the peripheral part of a bladder is shown. The *Nostoc* cells are about 6 μm in diameter. Drawings are based on electron microscopical observations. *BLO* bacteria-like organism, *CW* cell wall, *M* mitochondrion, *N* nucleus, *NC*, *Nostoc* cell, *PM* plasma membrane, *SM* symbiosome membrane, *V* vacuole

polarity and has a photosynthetically active region in the apical part of the cell exposed to light and air and a whitish-appearing storage region, containing many lipid droplets, in the basal part embedded in the soil surface. The center of the bladder is highly vacuolated. Schematic drawings of the compartmentation of *Geosiphon* are shown in *Fig. 16.17*. Ultrastructural observations show the *G. pyriformis* symbiosis as a system with very close contact between the partners. In fact, it is a symbiotic consortium of three organisms: (1) the fungus, supplying the consortium with inorganic nutrients like phosphate, trace elements, and water; (2) the cyanobacteria, supplying the consortium with carbohydrates by photosynthesis and, at least under some conditions, nitrogen compounds by N_2 fixation; (3) the "bacteria-like organisms" (BLOs), which are *Mollicutes*-related endobacteria, with yet unknown function.

Within the bladders, the cyanobacteria are located peripherally in a single, cup-shaped (often invaginated) compartment, the symbiosome. The *Nostoc* cells divide and are physiologically active as endosymbionts in this compartment. Within the cytoplasm of the fungus, glycogen granules exist as storage compounds. No dictyosomes are found; microtubules can rarely be observed. Fixation of the bladders during preparation for electron microscopy is often inadequate, probably due to the low cell wall permeability, but can be improved by using microwave acceleration.

Preparation of the *G. pyriformis* spores for electron microscopy (Schüßler et al. 1994) is even more difficult. This problem, caused by the thick spore wall being only slowly permeable to fixatives, also exists with other glomeromycotan species (Maia et al. 1993). Two main storage compounds occur inside the spores: lipid droplets of different sizes and "structured granules" that occupy about 25 % of the volume. The latter are discussed below with respect to element analysis. They show paracrystalline inclusions, as are also found in spores of some other glomeromycotan fungi. Small vacuoles are found in germinating spores and hyphae, often containing dark deposits. These are similar to the deposits in AM fungi and probably polyphosphate precipitates. The ultrastructure of the *Geosiphon* symbiosis was first studied by Schnepf (1964), and this was the crucial investigation leading to the theory of the compartmentation of the eukaryotic cell. The space between the symbiosome membrane and the wall of the enclosed *Nostoc* cells is only 30–40 nm wide and contains a layer of electron microscopically opaque and amorphous-appearing material which was originally assumed to be slime produced by the endosymbiont (Schnepf 1964). Later ultrastructural and confocal laser

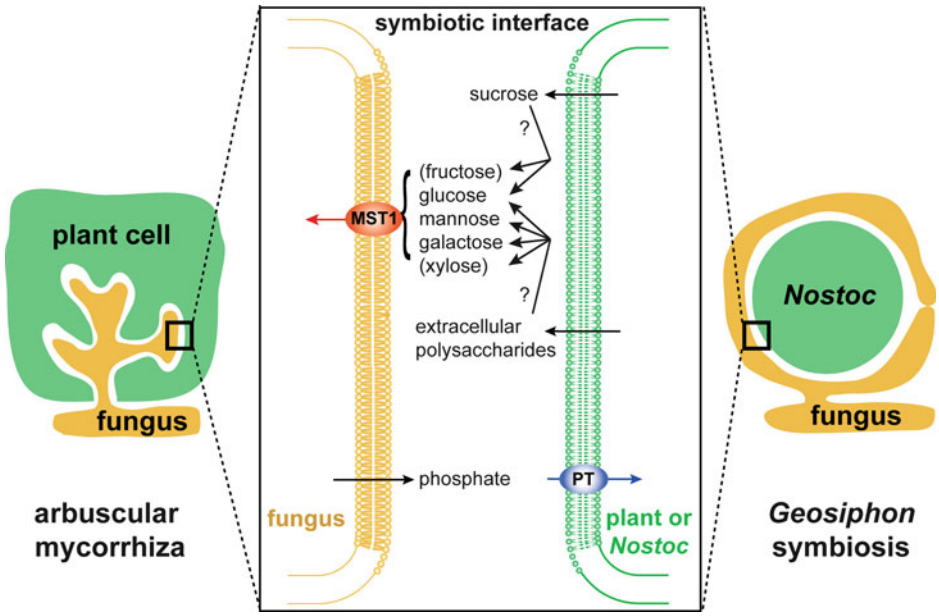


Fig. 16.18
The symbiotic interface and bidirectional nutrient flows in the *Geosiphon* symbiosis, in comparison with those in the arbuscular mycorrhiza (AM) (From Schüßler et al. 2006)

scanning microscopical (CLSM) studies by means of affinity techniques revealed that this amorphous layer inside the symbiosome contains chitin (Schüßler et al. 1996), confirmed by labeling with wheat germ agglutinin (WGA)-gold conjugates. Thus, the electron opaque layer within the symbiosome represents a "rudimentary" fungal cell wall, showing that the symbiosome membrane surrounding the *Nostoc* cells is homologous with a fungal plasma membrane.

Clear similarities exist between the fungal cell wall material present in the symbiosome space of the *Geosiphon* symbiosis and the thin arbuscular cell wall bordering the symbiotic AM fungus from the colonized plant cell: Both are electron dense after OsO_4 fixation, about 30–40 nm thick, and show the same amorphous structure and appearance. In general, the ultrastructural appearance of *G. pyriformis* is similar to that of AM fungi. Considering also the phylogenetic position of *G. pyriformis* and the known or proposed nutrient flows between the symbiotic partners, it has been suggested that the symbiotic interface in the AM and the *Geosiphon* symbiosis are homologous (Schüßler et al. 1996). The main difference between the symbioses is the relation of macro- and microbiont. In the *Geosiphon* symbiosis, the photoautotrophic partner (cyanobacterium) is the microsymbiont, whereas in the AM, it is the macrosymbiont (plant) (*Figs. 16.17*, *16.18*).

Element Composition and Distribution

It is not yet known why AM fungi cannot be cultured axenically. Also, there is little information available about their trace element requirements and general element composition. Considering the fact that these fungi supply the majority of land plants with inorganic elements, studies on the element composition and transport processes are interesting topics. We have used PIXE (proton induced X-ray emission) measurements to obtain the first indications of the macro- and microelement composition of the spores and symbiotic bladders. The element content of some subcellular compartments could be quantitatively measured and, by a differential approach, calculated. PIXE, combined with STIM (scanning transmission ion microscopy), allowed elemental concentrations to be absolutely quantified with a lateral resolution in the 1 µm range and with high accuracy and precision (Maetz et al. 1999a).

Studies on the *G. pyriformis* symbiotic bladders (Maetz et al. 1999b) showed that the fungal partner of the symbiosis while grown on a nutrient-poor solution (e.g., containing 1 µM phosphate) accumulated P in high concentrations (about 2 %), but not in the symbiosome. The P is probably stored as polyphosphate in the vacuoles, as for AM (and many other) fungi. High amounts of Cl (about 2.5 %) and K (about 8 %), which appear to play major roles in osmoregulation of the fungus, are found (all values given here are related to dry weight, ppm = µg/g DW). The symbiosome (including the cyanobacteria) contains only small amounts of these elements. This is in line with a presumed high concentration of monovalent ions in the fungal vacuoles. The macroelements Mg, S, and Ca and the microelements Fe, Mn, Cu, and Zn occur in concentrations comparable with those found in plants. The Se concentration is below 1 ppm. Mo is present within the symbiosome in very low amounts, compared with the rest of the bladder,

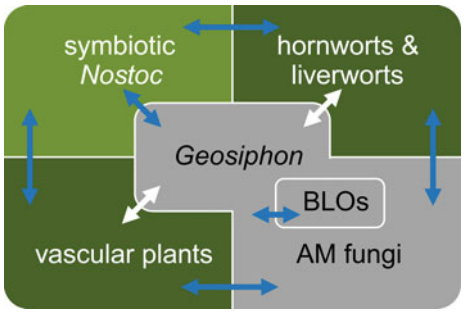


Fig. 16.19
A "symbiosis network" between cyanobacteria, fungi and plants. Associations or interactions which are highlighted with *white arrows* are hypothetical. The BLOs in glomeromycotan fungi play an unknown but probably important role in this network of intimate associations

although Mo is a constituent of nitrogenase, required for N_2 fixation of the cyanobacteria. Reasons for this might be that other Mo enzymes (e.g., nitrate reductase, sulfite oxidase) occur in sufficient amounts in the fungal cytoplasm or that Mo is located in the fungal vacuole. Mn and Ni, by contrast, are present in the symbiosome in much higher concentrations than in the rest of the bladder. Much of the Mn (approximately 50 ppm, which is comparable to values found in plant leaves) is probably contained in the water-cleaving Mn protein of photosystem II. Some may be from other enzymes, e.g., Mn-superoxide dismutase (SOD). A likely candidate for enzymes containing ~50 ppm Ni is cyanobacterial (or secreted fungal) urease; other Ni-containing bacterial enzymes are Ni-SOD and NiFe-hydrogenases.

Unpublished results on the element composition of the *Geosiphon* spores show that the structured granules (SGs), which are 4–6 µm in diameter, located each within a vesicle, together occupy about 25 % of the spore volume and contain most of the total P, K, and S. The S concentration of the spore cell wall is ~0.25 %, probably because of high protein content, as shown for an AM fungus (Bonfante and Grippiolo 1984). Compared with the bladders, Cl and K are concentrated within the spores in much lower amounts.

Signal Exchange Between Host and Cyanobacterium

It is not known what triggers the recognition process and the morphological changes during the establishment of the symbiosis. Microscopical studies give no hints for any chemotactic or otherwise directed growth toward the respective symbiosis partner, but the symbiosis-compatible *Nostoc* stage can be synchronized (Schüßler and Wolf 2005) to study this in more detail. Cells of particular strains of *N. punctiforme* can be incorporated by *Geosiphon*, resulting in the formation of functional symbioses. For other strains, although incorporated, the formation of symbiotic bladders is blocked at an early stage of development. Yet other *N. punctiforme* strains are not incorporated at all. Further evidence for a specific recognition process is the fact that, among the various developmental stages of *Nostoc*, only the early primordia, existing for ~3–12 h during the life cycle, are incorporated by the fungus. Not only is the physiological activity of the primordia different from the other stages of the *Nostoc* life cycle (Bilger et al. 1994) but so is the composition of the gelatinous envelope. When differentiating into "symbiosis-compatible" primordia, a mannose-containing slime is produced by the cells, whereas other sugars within the extracellular glycoconjugates can be detected only in earlier or later stages of the life cycle (Schüßler et al. 1997). The heterocysts (specialized N_2-fixing cells), differentiating at regular spacing along the filaments of the *Nostoc* primordia when grown under nitrogen limitation, always remain outside the fungal hypha during the incorporation process (Mollenhauer et al. 1996). They are not surrounded by a newly appearing mannose-containing glycoconjugate (Schüßler et al. 1997), also indicating a specific recognition of the early primordial cell surface by the fungus. Thus, alterations of extracellular glycoconjugates could be involved in partner recognition. Some unpublished data further indicate a lectin-mediated process.

Host-Cyanobiont Interactions Post Infection

Morphological Modifications of the Symbiosis Partners

The most obvious morphological change taking place after partner recognition is the formation of the *Geosiphon* bladder. Mature bladders represent large cells, which are coenocytic with the mycelium. They show a clear polarity, with the photosynthetically active symbiotic compartment (symbiosome) located in the apical part of the bladder (*Figs. 16.16*, *16.17*).

The symbiosome is derived from the invaginated plasma membrane of the fungus and contains the cyanobacteria, which are morphologically modified by increasing in volume about six- to eightfold compared with free-living vegetative cells. This is probably caused by the high osmotic pressure inside the bladders. In many plant symbioses, cyanobacteria are known to increase in size (Bergman et al. 1992a; Grilli Caiola 1992), probably as a reaction to the higher osmotic pressure of the surrounding medium. High NaCl concentrations are also known to cause an increase in volume of cyanobacteria (Erdmann and Schiewer 1984). For *Geosiphon* bladders, the iso-osmolar concentration of sorbitol was measured with oil-filled microcapillaries and determined to be 220–230 mM, corresponding to a turgor pressure (P) of about 0.6 MPa (Schüßler et al. 1995).

However, despite the increase in size, the *Nostoc* cells inside the *Geosiphon* bladder have an almost normal ultrastructure. They contain a high number of thylakoids and carboxysomes; one alteration is that the outer membrane is hardly recognizable

electron microscopically. Heterocysts are formed with the same frequency as in free-living colonies, but their cell wall is thinner in the symbiosis, possibly indicating a lower surrounding O_2 concentration.

N_2 and CO_2 Fixation and Transfer

^{14}C-tracer studies have shown that the *Geosiphon* bladders fix CO_2 both in light and in darkness, whereas the rate of CO_2 fixation in light is much higher (Kluge et al. 1991). In light, largely phosphate esters, poly-glucans, free sugars (including trehalose and raffinose), some amino acids, and organic acids trap ^{14}C. In darkness only malic and fumaric acids together with some amino acids appear as labeled products. High photosynthetic activity of the endosymbiotic *Nostoc* cells is also shown by photosystem II chlorophyll-fluorescence kinetics (Bilger et al. 1994). The symbiotic *Nostoc* cells achieve much higher steady-state quantum yields and electron transport rates when compared with free-living *Nostoc*.

The capability of N_2 fixation is indicated by the occurrence of heterocysts, and considerable nitrogenase activity is shown for the bladders (Kluge et al. 1992). In contrast to symbioses of *Nostoc* with plants, where usually a great increase of the heterocyst frequency indicates N_2 fixation as the major role of the cyanobacteria, in *Geosiphon* the relative heterocyst number does not change. Here, the major role of the endosymbiotic *Nostoc* is photosynthesis. However, matter exchange between the partners is still poorly investigated, and it is even possible that the second bacterial endosymbiont (BLO; ❯ *Fig. 16.14*), which is typical for many glomeromycotan fungi, may contribute to N_2 fixation.

For the endosymbiotic *Nostoc* cyanobacteria, all inorganic nutrients except N have to be provided by the fungus, as the cyanobacteria live intracellularly. As shown by electrophysiological experiments (unpublished), inorganic ions (nitrate, chloride) and small organic molecules (e.g., glycine, cysteine) lead to rapid, transient depolarization of the plasma membrane potential of the *G. pyriformis* bladders, indicating that these substances are actively taken up from the outside. By contrast, there are no changes in membrane potential if hexoses and larger amino acids are applied. In addition, metabolism of radioactively labeled hexoses by the bladders cannot be detected after the usual incubation times. Low cell wall permeability was considered the likely reason for the lack of uptake of monosaccharides. This theory is supported by observations showing that the presence of solutes with large molecule radii leads to irreversible cytorrhysis, i.e., collapse of the whole bladder including the cell wall, whereas in the presence of small solutes, plasmolysis occurred (or cytorrhysis was quickly reversed). This different transport behavior is presumably due to the selective permeability of the bladder wall.

By systematically using solutes with known molecular radii, it was shown that the limiting pore radius of the *G. pyriformis* bladder wall is approximately 0.5 nm, which, compared with other cell wall types, is very small (Schüßler et al. 1995). Such a pore size is too small for an efficient permeation by, e.g., hexose molecules from the outside, but it allows permeation of inorganic hydrated ions like phosphate. If the hyphal cell wall also has such a small pore size, the fungus would not be capable of saprobic acquisition of organic molecules such as glucose, sucrose, and larger amino acids. However, cell wall permeability is a complex topic, and the thin hyphae formed by AM fungi known as "branching absorbing structures" might possess different cell wall permeability.

Because AM fungi obtain up to 20 % of the plant-fixed CO_2, putatively as monosaccharides, the study of glomeromycotan sugar transporters that could play a role in C transfer from plants to AM fungi is important. Only one such glomeromycotan monosaccharide transporter had been characterized, and this (GpMST1) was from the *Geosiphon* symbiosis (Schüßler et al. 2006). This putatively symbiosome membrane-located transporter was demonstrated also to transport sugars potentially deriving from plant-cell-wall material (❯ *Fig. 16.18*). The GpMST1 sequence moreover provided valuable data for the isolation of homologues from other AM fungi and could eventually lead to the better understanding of C flows in the AM.

Ecological Importance

Why the Symbiosis Is Mutualistic

The fungus in the *Geosiphon* symbiosis belongs to the *Glomeromycota* (❯ *Fig. 16.12*) and is, like these, obligatorily symbiotic. It is not yet known why glomeromycotan fungi are not capable of nonsymbiotic life. Possibly it will be feasible in the future to develop special culture methods for in vitro growth of AM fungi, including *Geosiphon*. Generally, in nature, glomeromycotan fungi seem incapable of saprotrophic existence but are dependent on their symbiosis partners for C delivery. For *Geosiphon* bladders, it was shown that only small molecules can pass the cell wall (Schüßler et al. 1995). The narrow pores do not allow the uptake of hexoses or disaccharides from the outside, but permeation of inorganic ions like phosphate can occur. This might reflect the situation in nature. However, it is also very possible that the fine hyphae growing into the substrate show higher permeability. In any case, by incorporating *Nostoc*, the fungus obtains the required organic compounds.

Nostoc also benefits from the cooperation with the fungal host, which probably facilitates the supply of the endosymbiont with water, phosphate, and also CO_2. It is interesting that all inorganic nutrients, except N, have to be delivered by the fungus, since the cyanobacteria live intracellularly. It should also be kept in mind that the establishment of the *Geosiphon* symbiosis, as is usually true for AM, is strongly promoted by P limitation, which is a severe stress for the photobiont. The endosymbiotic *Nostoc* cells thus divide and grow faster and bigger than their free-living sisters. Preliminary studies moreover show that the intracellular cyanobacteria are protected against heavy metals, which

accumulate in the fungus (Scheloske et al. 2001). Therefore, as in the AM, the photobiont seems to be protected against abiotic stress factors in the *Geosiphon* symbiosis.

Evolutionary Implications with Ecological Meaning

Most vascular plant species form AM (Smith and Read 2008), including gametophytes and sporophytes of many ferns (Peterson et al. 1981) and *Lycopodiaceae* (Schmid and Oberwinkler 1993). Also, except for mosses, all groups of bryophytes contain species with AM associations (Ligrone 1988; Ligrone and Lopes 1989; Stahl 1949; Fonseca et al. 2009), indicating an early origin of the AM symbiosis.

In fact, the AM fungi have an ancient fossil record. Many of the oldest and best preserved ~400 MY old AM fungal fossils in association with plants are known from the Rhynie chert, radiometrically dated to the early Devonian (e.g., Remy et al. 1994; Dotzler et al. 2009). The oldest known fossils of what appear to be AM fungal spores and hyphae are from ~460 MY old Ordovician dolomite rock of Wisconsin (Redecker et al. 2000a), and it was concluded that terrestrial AM fungi already existed at a time when the land flora most likely consisted only of bryophyte-like "lower" plants.

From fossil cryptospore assemblages sharing characters with those of extant liverworts (found in what was eastern Gondwana; Rubinstein et al. 2010), it is estimated that land plants are more than 470 MY old (Early Middle Ordovician). The diversity of these assemblages implies an earlier, perhaps even Cambrian, origin of embryophytes. Early vascular plants already existed ~420 MY ago (Middle Silurian; Cai et al. 1996). A recent molecular clock study (Smith et al. 2010) suggested an origin of land plants around ~477 MY, but this dating in fact refers to the split between bryophytes and the remaining lineages, not the (presumably earlier) origin of the land plant lineage itself. Therefore, a minimum age of 420 MY for the liverwort-vascular plant divergence must be assumed, and bryophyte-like land plants were already present 510–470 MY ago.

Altogether, these data provide support for the hypothesis (Pirozynski and Malloch 1975) that AM fungi symbioses played a crucial role in the colonization of the land by plants, evolving from a partnership between two aquatic types of organisms, algae, and "oomycetous" fungi (the authors recognized the difference between AM fungi and other "phycomycetes," and thus interpreted them as "oomycetes," which are nowadays known not to be fungi), as the initial step of land plant evolution. A mycotrophic lifestyle could have been essential for an efficient supply of plants with water and nutrients from the soil (Malloch et al. 1980; Marschner and Dell 1994). However, molecular clock estimates always date the origin of the AM fungal lineage to be at least 50, possibly more than 200 MY earlier than that of land plants. If this holds true, it implies that there were other types of associations formed by AM fungi before land plants existed, whether saprobically, parasitically, or already mutualistically. *Geosiphon pyriformis*, representing a symbiotic association between a glomeromycotan fungus and a photoautotrophic prokaryote, may reflect such an ancestral partnership, and thus, indirectly but substantially, supports the hypothesis regarding pre-*Embryophyta* associations of AM fungal predecessors (Pirozynski and Malloch 1975). It is very plausible to assume that at the beginning of terrestrial plant life, also other associations between glomeromycotan fungi and photoautotrophic organisms (like the ubiquitous cyanobacteria) existed. The present knowledge regarding AM fungi and AM symbiosis evolution was recently discussed and reviewed (Schüßler and Walker 2011).

In summary, glomeromycotan fungi may have adapted to symbiotic life more than 500 MY ago. Without fossil support, this is speculative, but *G. pyriformis* clearly confirms the ability of glomeromycotan fungi to form symbioses with even prokaryotic photoautotrophic organisms. Therefore, cyanobacterial symbioses formed by glomeromycotan fungi could have been an ecologically important step for the colonization of the land habitat.

Arbuscular mycorrhizal fungi form symbioses with most land plants, and individual AM fungi can be symbiotic with widely divergent photoautotrophs such as hornworts and vascular plants (Schüßler 2000). The genetic base for these interactions is highly conserved (Wang et al. 2010). One can speculate that there might even be very ancestral symbiotic mechanisms in the AM that can be found in the *Geosiphon-Nostoc* symbiosis. There are some very fundamental and conserved mechanisms of plant-microorganism interactions present among the different AM(−like) associations. When conducting ecophysiological studies involving plants, it is important to consider that in nature the mycorrhizal fungal partners are the main facilitators of nutrient uptake, rather than the plant roots alone. If, as is thought, the mechanisms of nutrient acquisition by land plants coevolved since their origin with the AM fungi, ecologically and economically important questions might be answered by using the *Geosiphon* symbiosis as a model.

A Network Between Fungi, Cyanobacteria, and Plants?

Against the above described evolutionary background, the interesting question arises as to whether *G. pyriformis* itself can act as a fungal partner to form AM. Unpublished results showed that *Geosiphon* rDNA can be PCR amplified from plant roots and bryophytes growing in the natural habitat of *Geosiphon*. However, it cannot be completely ruled out that the sensitive nested PCR approach is detecting tiny amounts of DNA from externally attached hyphae. Future studies at sites where the cyanobacteria symbiosis of *Geosiphon* never occurs will have to show whether *Geosiphon* is indeed forming an AM. If this were to be the case, a complex network of ecological importance may be imagined (*Fig. 16.19*).

The molecular probes to screen for the occurrence of *Geosiphon* in the soil and plant roots have been developed.

If *Geosiphon* indeed forms AM with plants, a complex network of biotic interactions would exist in the natural habitat. Within such a network, symbiotic *Nostoc* could be exchanged between *Geosiphon* and bryophytes, and *Geosiphon* could simultaneously form endosymbiosis with *Nostoc* and AM with plants, thus, e.g., transporting and delivering N_2 fixed by the cyanobacteria to the plants. Unfortunately, funding applications have been rejected, with the consequence that laboratory cultures are no longer available.

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