Cyanobacterial Symbioses

23

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Contents

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Summary

Cyanobacteria form symbiotic partnerships with a wide range of eukaryotic hosts including fungi, plants and animals such as sponges, ascidians and corals. They provide the host with fixed nitrogen and fixed carbon, and in return occupy relatively protected environments free from predation and environmental extremes. As well as being photoautotrophs, many cyanobacteria are capable of heterotrophy, enabling them to occupy symbiotic structures, such as the roots of plants, that receive little or no light and where photosynthetic hosts can supply them with fixed carbon. In all but a few cases cyanobacterial symbionts are capable of independent growth, but they frequently show significant morphological and physiological modifications when in symbiosis. Many cyanobacterial symbionts fix N_2 in specialised cells known as heterocysts and in many symbioses, notably those with plant hosts, the frequency of heterocysts is greatly elevated, as is the rate of N_2 fixation. A number of cyanobacterial symbioses are of major environmental significance as suppliers of fixed nitrogen to their surroundings. Some, such as the diatoms, can reach enormous populations in the oceans, whereas moss epiphytic associations are abundant in boreal forests, and cyanolichens are abundant in harsh environments where there are few other sources of fixed nitrogen.

23.1 Introduction

Cyanobacteria are found in symbiosis with a remarkable variety of hosts including plants, fungi, sponges and protists. In the majority of these symbioses the cyanobiont's contribution to the partnership is the products of N_2 fixation, enabling the hosts, such as plants and lichens, to occupy N limited environments. However, being photoautotrophs, cyanobacterial symbionts (cyanobionts) are also capable of supplying fixed carbon to non-photosynthetic hosts such as the fungi of lichens and *Geosiphon pyriformis*. Cyanobionts may also help to protect the host from excessive sunlight (e.g. sponges) or grazing (e.g. ascidians and isopod crustaceans). What the cyanobacteria gain is often less obvious, although protection from environmental extremes, predation and competition are all likely benefits. Photosynthetic hosts can also provide fixed carbon to the cyanobiont and this capacity for heterotrophy enables many cyanobionts to grow in host structures, such as the roots of cycads or the stem glands of *Gunnera*, that receive little or no light. All hosts are capable of independent growth if provided with the necessary nutrients, as are all cyanobionts, with the exception of those in *Azolla* and some diatoms. Within the wide variety of cyanobacterial symbioses there seems to be no correlation between the presumed evolutionary age of the symbiosis and the location of the cyanobiont (e.g. inter- or intra-cellular) within the host, its mode of transmission, or its importance to host well-being (Usher et al. 2007). In other words, the degree of integration between host and cyanobiont, and the mode of cyanobiont transmission are not good indicators of the evolutionary age of the symbiosis or its importance to the host.

A wide variety of cyanobacteria, both unicellular and filamentous, forms symbiotic associations. Perhaps the most common cyanobionts are from the genus *Nostoc* and possess two important characteristics – they fix N_2 in specialised cells known as heterocysts and they produced motile filaments known as hormogonia. The heterocyst provides the necessary microoxic environment for the functioning of the enzyme nitrogenase, which performs biological N_2 fixation and is highly sensitive to oxygen (Golden and Yoon 2003; Zhang et al. 2006; Flores and Herrero 2010). Unicellular or filamentous non-heterocystous N_2-fixing cyanobacteria have to employ alternative strategies to protect nitrogenase, such as the temporal separation of N_2 fixation and oxygenic photosynthesis. The hormogonium provides a motile phase in the otherwise sessile *Nostoc* life cycle and serves both as a means of dispersal and as the infective agent in many of the cyanobacterial symbioses with plants and fungi (Meeks et al. 2002; Meeks and Elhai 2002; Meeks 2009). Many plant hosts secrete chemical signals that both stimulate hormogonia production and serve as chemoattractants to guide the hormogonia to the symbiotic structures. Once infected, some, and perhaps all plant hosts secrete hormogonia-repressing factors to ensure that the cyanobiont returns to vegetative growth and produces heterocysts to fix N_2 for the host.

This chapter deals with the literature from 2000 onwards. For coverage of the earlier literature the reader is directed to Adams (2000) and the many reviews and chapters listed here. Earlier research on cyanobacterial symbioses was largely concerned with the more experimentally amenable of the plant associations such as those with *Gunnera*, *Azolla* and bryophytes such as hornworts and liverworts. More recently there has been increasing interest in associations such as sponges and mosses.

23.2 The Symbioses and Their Environmental Impact

23.2.1 Plants

Cyanobacterial-plant symbioses are ancient associations believed to have evolved around 500 million years ago (Raven 2002a, b; Bergman et al. 2008a, b), a hypothesis supported by the discovery of fossil evidence of cyanobacteria

inside 400 million year old land plants (Taylor and Krings 2005; Krings et al. 2009); these cyanobacteria were non-heterocystous and probably more closely resembled Oscillatoriales rather than the Nostocales that typically enter into existing plant associations. Warm and moist environments, supporting close association of plants and cyanobacteria, probably stimulated the evolution of cyanobacterial symbioses (Usher et al. 2007). These conditions are thought to have favoured enhanced plant growth, thereby increasing the demand for N. Additionally, for hormogonia (the infective agents in most cyanobacteria-plant symbioses; Sect. 23.4.1.2), to remain motile they require the presence of some moisture, such as a thin film of water (Usher et al. 2007). Although in most cases the cyanobiont is still capable of growth away from the host, an exception is the water fern *Azolla* in which the adaptations of the cyanobiont are more extreme and it is no longer capable of independent growth, indicating that some biological feature critical for the free-living state has been lost during the millions of years of co-evolution with its host. The cyanobiont might even be evolving towards being a N_2 fixing organelle in a manner akin to that believed to have given rise to the chloroplast (Ekman et al. 2008; Ran et al. 2010).

23.2.1.1 Loose Associations

Although most of the symbioses described in this chapter involve cyanobionts living within the tissues or the cells of the host, there are many looser associations in which cyanobacteria grow as epiphytes on the surface of plants. With the exception of the cyanobacteria-moss epiphytic associations, which are dealt with later, these associations are mostly poorly studied. They are probably common, although the degree of benefit obtained by the epiphytic cyanobacterium and the plant is often unclear.

Epiphytic growth of N_2 fixing *Nostoc*, *Gloeotrichia*, *Anabaena*, *Calothrix* and *Cylindrospermum* has been reported for rice plants and duckweed (see Adams 2000) and the unicellular *Chamaesiphon* spp. and *Xenococcus kerneri*, which have not yet been checked for possible N_2 fixation, are common on epilithic algae and submerged mosses (Lindstrøm et al. 2004; Kučera et al. 2005). Cyanobacteria are also common epiphytes on the pneumatophores of mangroves (Steinke et al. 2003). In rice fields in Spain the macroalga *Chara vulgaris* harbours N_2 fixing epiphytic cyanobacteria belonging to the heterocystous genera *Calothrix*, *Nostoc* and *Anabaena* (Ariosa et al. 2004). *Chara* is found in rice fields world-wide and is generally thought to be a weed, but its N_2 fixing epiphytic cyanobacteria may contribute to soil fertility (Ariosa et al. 2004). Various cyanobacteria, including *Nostoc*, *Scytonema* and *Calothrix*, have also been found on the aerial roots of the epiphytic orchids *Acampe papillosa*, *Phalaenopsis amabilis* and *Dendrobium moschatum* and the substrate roots of *A. papillosa* and *D. moschatum* although it is unclear if the orchids benefit from fixed N produced by the cyanobacteria (Tsavkelova et al. 2001, 2003a, b). A potentially endophytic cyanobiont, resembling the unicellular *Dactylococcopsis acicularis*, has been reported in the roots of the orchid *Spathoglotis plicata* (Untari et al. 2009).

Epiphytic growth of cyanobacteria is also common in the marine environment. For example, the chlorophyll *d*-containing cyanobacterium *Acaryochloris marina* is found as an epiphyte on the marine red macroalga *Ahnfeltiopsis flabelliformis* (Murakami et al. 2004) and on green and brown marine macroalgae (Ohkubo et al. 2006). Another marine red macroalga, *Acanthophora spicifera*, can become covered by epiphytic *Lyngbya* (Fong et al. 2006). *A. spicifera* formed blooms on some Eastern Pacific reefs following coral mortality resulting from the 1997–1998 El Nino Southern Oscillation. The alga lacking the cyanobacterial epiphyte is highly palatable to herbivores, but the cyanobacterium greatly reduces herbivory, presumably by the production of chemical defences, and this increases the ability of the alga to become dominant (Fong et al. 2006).

Lyngbya spp. are also found as epiphytes on seagrasses in Florida Bay where addition of P stimulates their growth and that of co-occurring red algal epiphytes (Armitage et al. 2006; Frankovich et al. 2009). Bacterial epiphytes on seagrasses such as *Thalassia testudinum* may obtain organic carbon from cyanobacterial and algal epiphytes rather than from the seagrass itself (Williams et al. 2009). Cyanobacteria are common epiphytes on seagrasses, and in highly oligotrophic seas such as the Gulf of Elat they can make significant contributions to the N required for primary productivity in the seagrass beds (Pereg-Gerk et al. 2002). Cyanobacterial epiphytes on the leaves of three seagrasses *Thalassodendron ciliatum*, *Thalassia hemprichii* and *Cymodocea rotundata* from two Kenyan coastal sites show enough distinct differences between the seagrass species to suggest that there may be some host specificity, particularly in *C. rotunda* (Uku et al. 2007). On the same seagrasses low nutrient levels favour the growth of cyanobacterial over algal epiphytes (Uku and Bjork 2001). Heterocystous cyanobacteria such as *Calothrix* and *Anabaena* and other potential N_2 fixers may enable *C. rotunda* to maintain a rapid growth rate at a low-nutrient, N-limited site, where seagrasses lacking these cyanobacteria are disadvantaged. In this way N_2 fixation by epiphytic cyanobacteria may contribute to the productivity of seagrass beds (Hamisi et al. 2009).

Compared with the leaves of aquatic plants, the leaf surface (phyllosphere) of land plants is a much harsher environment for cyanobacteria, but they can be found in the phyllosphere in tropical rainforests where the humidity is high. For example, in a Costa Rican lowland rainforest heterocystous *Nostoc*, *Fischerella* and *Tolypothrix* are found on leaf surfaces, often in association with epiphytic bryophytes

(Fürnkranz et al. 2008). Although found at comparatively low abundance, these cyanobacteria are the major component of the N_2 fixing bacterial community and may provide significant N input into this rainforest ecosystem. However, not all epiphytic cyanobacteria are beneficial to the "host". For example, *Brasilonema octagenarum* strain UFV-E1 (Scytonemataceae) forms a dense mat on the surface of the leaves of *Eucalyptus grandis* (Aguiar et al. 2008). The cyanobacterial mat blocks light, causing a reduction in photosynthesis in the leaves, and interfering with stomatal gas exchange, so decreasing CO_2 assimilation.

23.2.1.2 Bryophytes (Mosses, Hornworts and Liverworts)

The bryophytes, encompassing the liverworts (Hepaticae), the hornworts (Anthocerotae) and the mosses (Musci), are small, non-vascular land plants, some of which form epiphytic or endophytic (Figs. 23.1 and 23.2) associations with cyanobacteria (Adams 2002a, b; Meeks 2003; Solheim et al. 2004; Adams et al. 2006; Adams and Duggan 2008; Bergman et al. 2007a, 2008a), primarily of heterocyst-forming genera *Nostoc*, *Stigonema* and *Calothrix*. In the mosses the cyanobacteria are mostly epiphytic, often being found between the stem and the leaf (Solheim and Zielke 2002; Solheim et al. 2004; Gentili et al. 2005), with the exception of two *Sphagnum* species in which they occupy water-filled, dead (hyaline) cells, where they are thought to be protected from the acidic bog environment (Solheim and Zielke 2002). Cyanobacteria growing on the moss leaf surface are thought to be protected by alkaline substances secreted by the leaf (Belnap 2001). In *Sphagnum*, the acidity of the bog environment may be the factor that determines whether cyanobacteria grow epiphytically (higher pH) or intracellularly within hyaline cells (lower pH; Solheim and Zielke 2002). These moss associations with N_2 fixing cyanobacteria can supply most of the combined nitrogen in local ecosystems in the Arctic, the Antarctic and boreal forest regions (Zielke et al. 2002, 2005; Solheim and Zielke 2002; Nilsson and Wardle 2005; DeLuca et al. 2008; Stewart et al. 2011). Examples of other forest systems where they may be a major source of biologically-derived nitrogen include ones from tropical forests (Cusack et al. 2009) and New Zealand (Menge and Hedin 2009). This topic is discussed further in Chap. 10.

Fig. 23.1 The liverwort *Blasia pusilla* collected from the wild, showing the dark *Nostoc* colonies (~0.5–1.0 mm in diameter) bordering the thallus midrib. (Reproduced with permission from Adams 2000)

Mosses

Cyanobacteria-moss associations may be especially important in boreal forests where up to 80% of the ground cover can consist of the feather moss *Pleurozium schreberi* with its epiphytic cyanobacteria (Zackrisson et al. 2004; Gentili et al. 2005; Nilsson and Wardle 2005; Lagerström et al. 2007; DeLuca et al. 2007, 2008). Indeed, *P. schreberi* is one of the most common mosses on earth (DeLuca et al. 2002) and in boreal forests feather moss growth can exceed that of trees (Bond-Lamberty and Gower 2007). The mosses *Hylocomium splendens* and *Ptillium crista-castrensis* also associate with N_2-fixing cyanobacteria (Solheim et al. 2004; Houle et al. 2006; Zackrisson et al. 2009) and the moss *Sphagnum capillifolium* has even higher rates of N_2 fixation than *Pleurozium schreberi*, even when the two occur at the same site (Markham 2009). Recent work in old growth forests in British Columbia has shown that epiphytic moss-cyanobacteria associations may show nitrogen fixation rates even greater than those of moss carpets on the forest floor (Lindo and Whiteley 2011).

In alpine and arctic heath tundra in Sweden *Pleurozium schreberi* and *Hlyocomium splendens* are usually responsible for less than 5% of the ground cover, yet under patches of the common juniper this can be as high as 60–80%. These moss carpets have N_2 fixation rates of 150 µmol acetylene reduced m^{-2} day^{-1}, 10–15 times higher than in the open heath (DeLuca and Zackrisson 2007). This elevated N_2 fixation rate may result from the ability of the junipers to use their extensive root systems to scavenge P, resulting in raised P levels beneath the shrubs as a result of litter deposition. The presence of these moss "islands" in this alpine tundra can result in levels of N_2 fixation as high as 1.4 kg N ha^{-1} $year^{-1}$ (DeLuca and Zackrisson 2007). However, the N fixed by cyanobacteria epiphytic on feather moss may have a relatively low availability to the local ecosystem because the mosses are highly efficient

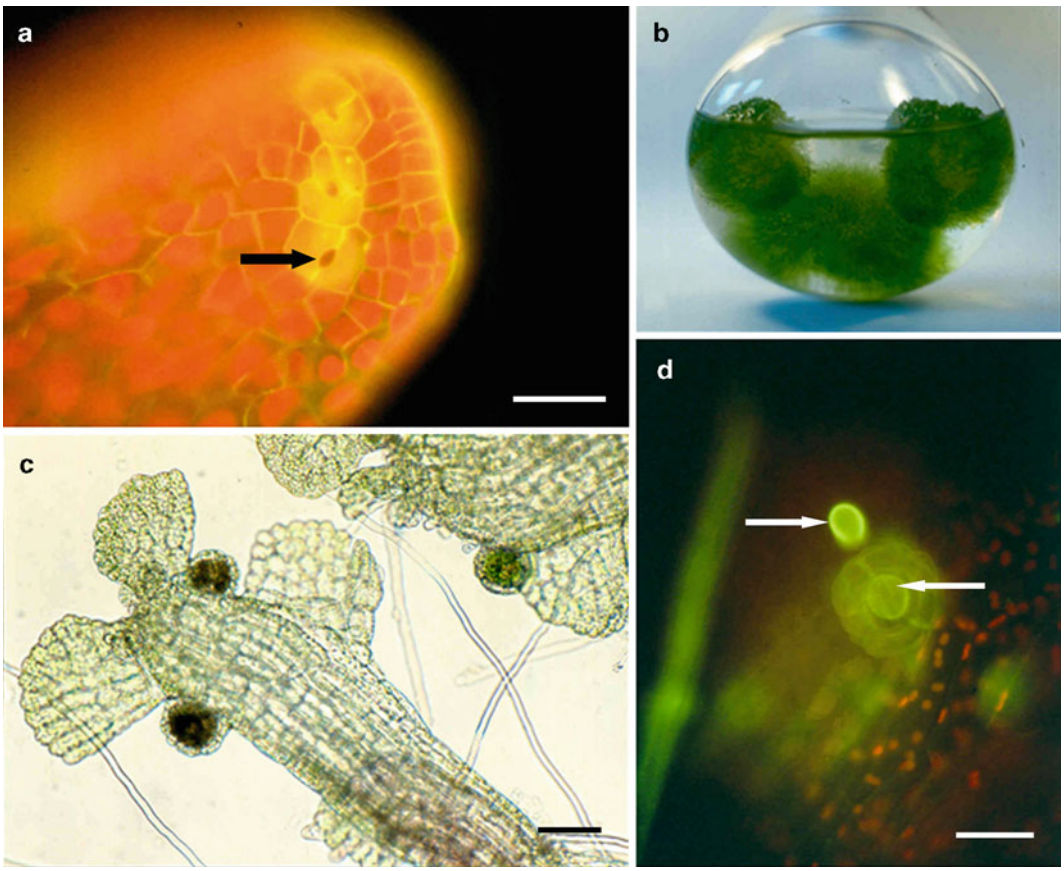

Fig. 23.2 The liverwort and hornwort symbioses. (**a**) Fluorescence micrograph of the hornwort *Phaeoceros* stained with calcofluor, showing the slit-like entrances (one of which is arrowed) through which hormogonia gain entry to the slime cavities beneath. (**b**) View of the underside of an Erlenmeyer flask containing the liverwort *Blasia pusilla* grown free of cyanobacteria in shaken liquid medium. (**c**) *Blasia pusilla* growing in liquid culture showing three auricles 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 uninfected *Blasia* stained with calcofluor. A single auricle can be seen with one inner (lower arrow) and one outer (upper arrow) slime papilla. Bars 50 μm (Photographs (**a**) and (**d**) courtesy of S. Babic. (**a**) and (**d**) reproduced with permission from Adams 2000; (**b**) reproduced with permission from Adams 2002a; (**d**) reproduced with permission from Adams and Duggan 1999)

at retaining this N and the decomposition rate of dead feather moss tissue is very low (Lagerström et al. 2007).

N_2 fixation by cyanobacteria-moss associations is greatly influenced by existing environmental factors, such as water availability, and may be adversely affected by future changes such as ozone depletion and the resulting increases in UV-B radiation. For example, N_2 fixation rates in cyanobacteria-moss associations in the arctic are greatly reduced by 3–6 years exposure to artificially enhanced UV-B radiation, equivalent to a 15% depletion of the ozone layer (Solheim et al. 2002, 2006). Prolonged drought can also result in a decline in the N_2 fixation capacity of cyanobacteria-moss associations, whereas persistent moisture results in an increase (Gundale et al. 2009). This probably explains the frequent reports of decreased N_2 fixation rates in mid-summer when conditions are at their driest (DeLuca et al. 2002; Zackrisson et al. 2004).

N_2 fixation in cyanobacteria-moss associations is very sensitive to external combined N. For example, fertilization with ammonium nitrate can reduce or eliminate N_2 fixation (Zackrisson et al. 2004; DeLuca et al. 2007; Gundale et al. 2011) and reduce colonization of moss shoots by cyanobacteria (DeLuca et al. 2007). However, sensitivity to external N input varies between mosses. For example, the cyanobacteria-*P. schreberi* association seems to be more sensitive to external N input than the *H. splendens* association (Zackrisson et al. 2009). Deposition of canopy throughfall N onto moss carpets can also inhibit cyanobacterial N_2 fixation (DeLuca et al. 2008). This inhibition by available nitrogen may explain the gradual increase in N_2 fixation rates following recovery from fire, a process which may take hundreds of years (Zackrisson et al. 2004; DeLuca et al. 2008). Support for this comes from transplantation of moss carpets from early secondary successional boreal forest sites (up to 101 years since

the last fire and with high levels of available N) to late successional sites (241–356 years since the last fire and with low levels of N). The low N, late successional sites had high rates of N_2 fixation and high levels of cyanobacterial colonization of moss shoots, but moss carpets transplanted from these sites to early successional sites, with high levels of available N, showed a decline in N_2 fixation rates and cyanobacterial colonization after 12 months (DeLuca et al. 2007). Conversely, transfer of late successional moss carpets to early successional sites resulted in decreased N_2 fixation rates and a decrease in cyanobacterial colonization. Other aspects of the interactions between cyanobacteria and these feather mosses are discussed in Sect. 10.4.6.

Hornworts and Liverworts

In the hornworts, of which 13 genera have been described (Duff et al. 2007), endophytic cyanobacterial associations are ubiquitous (Renzaglia et al. 2007) and new ones, such as *Nothoceros superbus*, are still being found (Villarreal et al. 2007). By contrast, of more than 340 liverwort genera only four form cyanobacterial associations, two of which (*Blasia* and *Cavicularia*) are endophytic and two (*Marchantia* and *Porella*) epiphytic (Adams et al. 2006; Adams and Duggan 2008). The flattened gametophyte thallus of liverworts and hornworts is a few centimetres in length and symbiotic colonies can be seen as dark spots up to 0.5 mm in diameter (Fig. 23.1). The thallus is attached to the substrate by root-like rhizoids. Liverworts such as *Blasia* and hornworts such as *Anthoceros* and *Phaeoceros*, make excellent laboratory models for cyanobacteria-plant symbiosis because of the ease with which the host plant can be grown, free of its symbionts, in shaken liquid culture, and the symbiosis re-formed with the original or with novel cyanobacteria (Fig. 23.2b; Meeks 2003; Duckett et al. 2004; Adams and Duggan 2008).

23.2.1.3 Gymnosperms (Cycads)

Between 250 and 65 million years ago the cycads dominated the Earth's forests, but today their distribution is limited to subtropical and tropical regions of mostly the southern hemisphere, including Australia and South Africa (Brenner et al. 2003; Vessey et al. 2005). They are the most primitive of today's seed plants (gymnosperms), consisting of approximately 250 species within the order Cycadales (Vessey et al. 2005; Bergman et al. 2007a). These evergreen, palm-like plants vary in height from a few tens of centimetres to 20 m, with a trunk and a large tap root from which may develop two additional root types: lateral and coralloid. Coralloid roots (so-called because of their coral-like appearance; Fig. 23.3a) are produced by all cycad species and show negative geotropism, growing sideways and upwards towards the soil surface; they become infected with N_2 fixing cyanobacteria, primarily of the genus *Nostoc* (Costa and Lindblad 2002; Lindblad and Costa 2002; Vessey et al. 2005; Bergman et al. 2007a), which are visible as a dark blue-green band between the inner and outer cortex (Fig. 23.3b). Nitrogen fixation in cycads can contribute up to 18.8 kg N ha^{-1} $year^{-1}$ to the local N economy (see: Rai et al. 2000; Vessey et al. 2005).

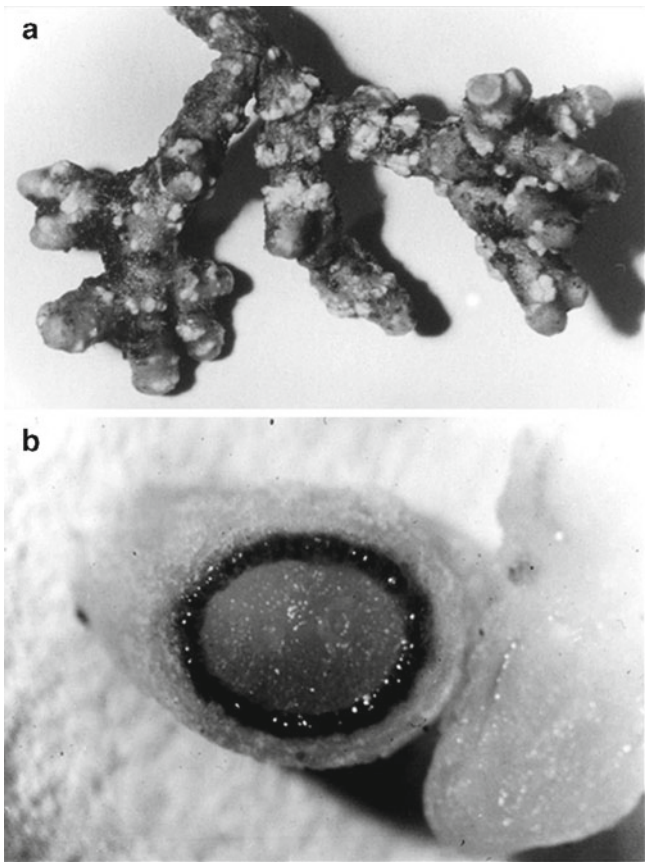

Fig. 23.3 The cycad-*Nostoc* symbiosis. (**a**) Cycad coralloid root, which is the site of cyanobacterial infection. (**b**) Transverse section of the root showing the dark cyanobacterial band between the inner and outer cortical layers of the root ((**a**) Reproduced with permission from Lindlbad et al. 1985; (**b**) reproduced with permission from Rai et al. 2000)

The cyanobionts of some cycad coralloid roots have been shown to produce the neurotoxic non-protein amino acid β-methylamino-L-alanine (BMAA; Cox et al. 2003) which may act as a deterrent to herbivory in cycads. BMAA was later shown to be produced by all known groups of free-living cyanobacteria (Cox et al. 2005; Banack et al. 2007). This neurotoxin was thought to be responsible for 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, who ingested the toxin through eating flying foxes (fruit bats) which had themselves eaten cycad seeds containing BMAA (Cox et al. 2003; Banack et al. 2006). However, this theory has remained controversial, not least because of difficulties in reliable separation and detection of BMAA. Some groups have confirmed the presence of BMAA

Fig. 23.4 *Gunnera manicata*. (**a**) Young plant with two large flower spikes. Inset: Red pigmented fronds cover the crown of the plant (hidden in the large image), where new leaves and new stem glands develop. (**b**) Vertical cross-section of a rhizome. Cyanobacterial colonies (0.5–2 cm in diameter) can be seen as green patches around the periphery of the rhizome. New leaves will develop in the region between the two leaf petioles at the top of the image, which is an area covered by red fronds (see inset in **a**). New stem glands form close to the base of each newly-developing leaf petiole and subsequently become infected by *Nostoc*. (Photos: Owen Jackson)

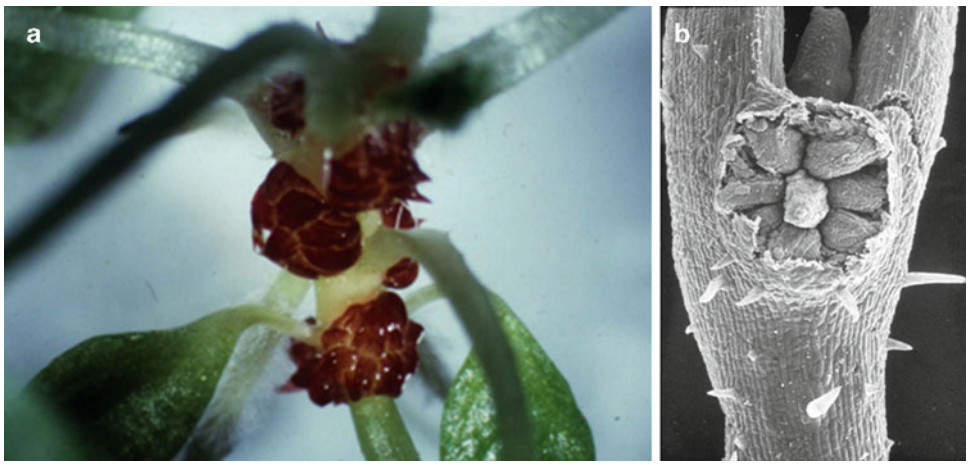

Fig. 23.5 *Gunnera* stem glands. (**a**) *Gunnera* seedling showing the red stem glands at the base of the leaf petioles. The glands are the entry point for cyanobacteria. (**b**) Scanning electron micrograph of a *Gunnera chilensis* gland showing the arrangement of papillae. Hormogonia gain entry into the internal stem gland tissue by migrating down the channels between the papillae. ((**a**) Reproduced with permission from Adams et al. 2006; (**b**) reproduced with permission from Bergman et al. 1992)

in cyanobacteria and cycad seeds (Esterhuizen and Downing 2008; Spáčil et al. 2010). However, contradictory results have been obtained when samples have been analysed by LC-MS/MS without prior derivatisation of samples (Rosén and Hellenäs 2008; Li et al. 2010; Krüger et al. 2010).

23.2.1.4 Angiosperms (*Gunnera*)

The symbiosis between *Nostoc* and *Gunnera* is unique for two reasons – it is the only symbiosis between an angiosperm (flowering plant) and a cyanobacterium, and it is the only one in which the cyanobiont is intracellular (Bergman 2002; Bergman and Osborne 2002; Bergman et al. 2007a). The cyanobiont is found inside mucus-secreting glands on the plant stem at the base of each leaf petiole (Figs. 23.4 and 23.5). The *Gunnera* genus consists of around 50 species that vary in size from creeping forms with leaves 1–10 cm across, to rhizomatous plants with leaves several metres across (such as *G. manicata*; Fig. 23.4a). The *Gunnera* cyanobiont may constitute as little as 1% of the plant mass yet can supply the entire N requirements of even

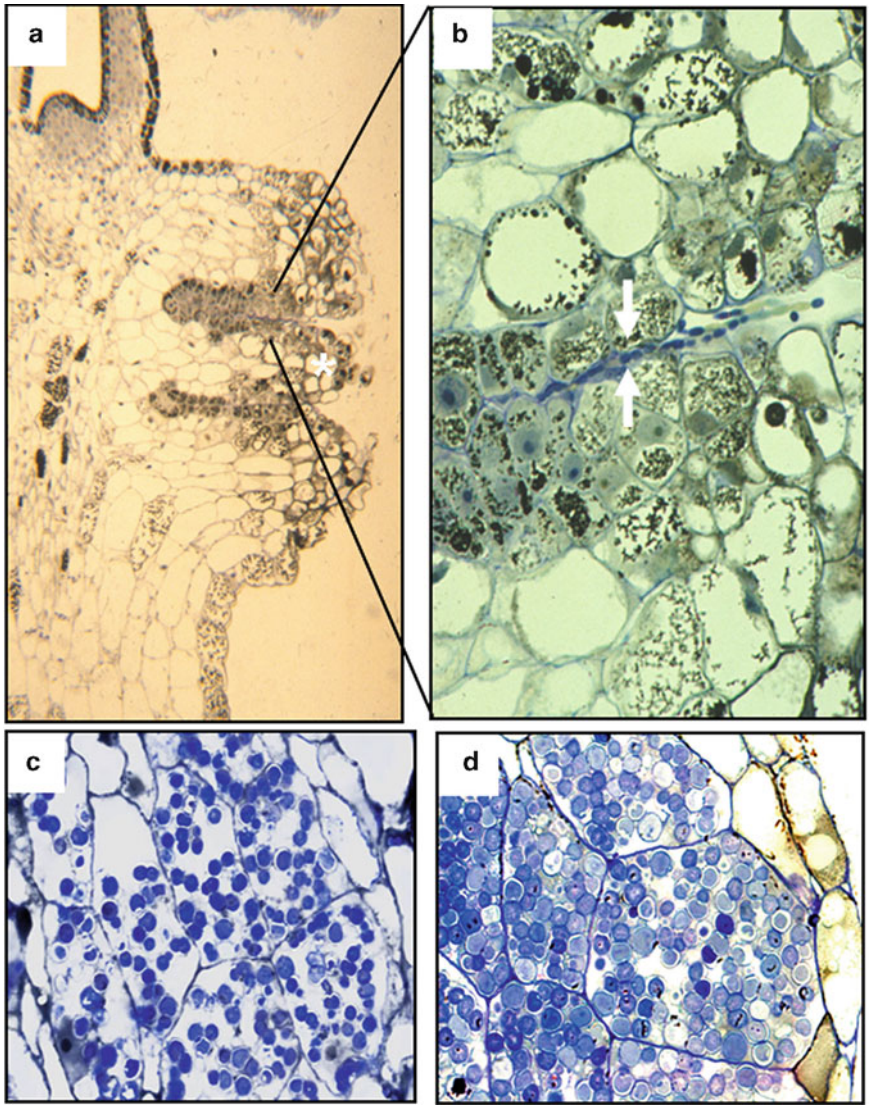

Fig. 23.6 *Gunnera* stem gland structure and infection. (**a**) Cross section of the stem gland showing three papillae separated by the channels that provide the route of infection into the gland tissues. (**b**) Close-up of one of the channels in (**a**) showing hormogonia (stained blue; arrows) migrating towards the inner parts of the gland. (**c**) *Gunnera* cells infected with cyanobacterial filaments (stained blue). At this early stage the filaments have a very low frequency of heterocysts, whereas at later stages (**d**) the heterocyst frequency increases greatly. (Reproduced with permission from Johansson and Bergman 1992)

the largest plants (Bergman et al. 2007a). The plants have a fossil record dating back 70–90 million years, making them the oldest of the angiosperms (Wikström et al. 2001; Raven 2002a). They were once only found in warm, wet equatorial regions such as South America, South East Africa, Madagascar and the Philippines, but now commonly appear in temperate climates such as Northern Europe, in suitably wet conditions (Osborne and Sprent 2002). The ecology, taxonomy and biogeography of the plant is reviewed elsewhere (Wanntorp and Wanntorp 2003; Fuller and Hickey 2005) (Fig. 23.6).

The *Nostoc-Gunnera* symbiosis appears to be mutually beneficial, in that the plant receives fixed nitrogen from the cyanobacterium (Uheda and Silvester 2001; Bergman and Osborne 2002; Bergman 2002), and while benefits to the cyanobacterium are less clear, it is likely that it is provided with an uncompetitive ecological niche, protection from predation and environmental extremes including desiccation (Badger et al. 2006), and also with fixed carbon from the plant (Black et al. 2002), possibly in the form of fructose (Parsons and Sunley 2001; Ekman et al. 2006). The relationship also appears to be facultative, in that both the plant and the cyanobiont can be cultured separately (Chiu et al. 2005). However, *Gunnera* only thrives when the cyanobiont is present; indeed all *Gunnera* plants in the wild contain *Nostoc* as an intracellular cyanobiont.

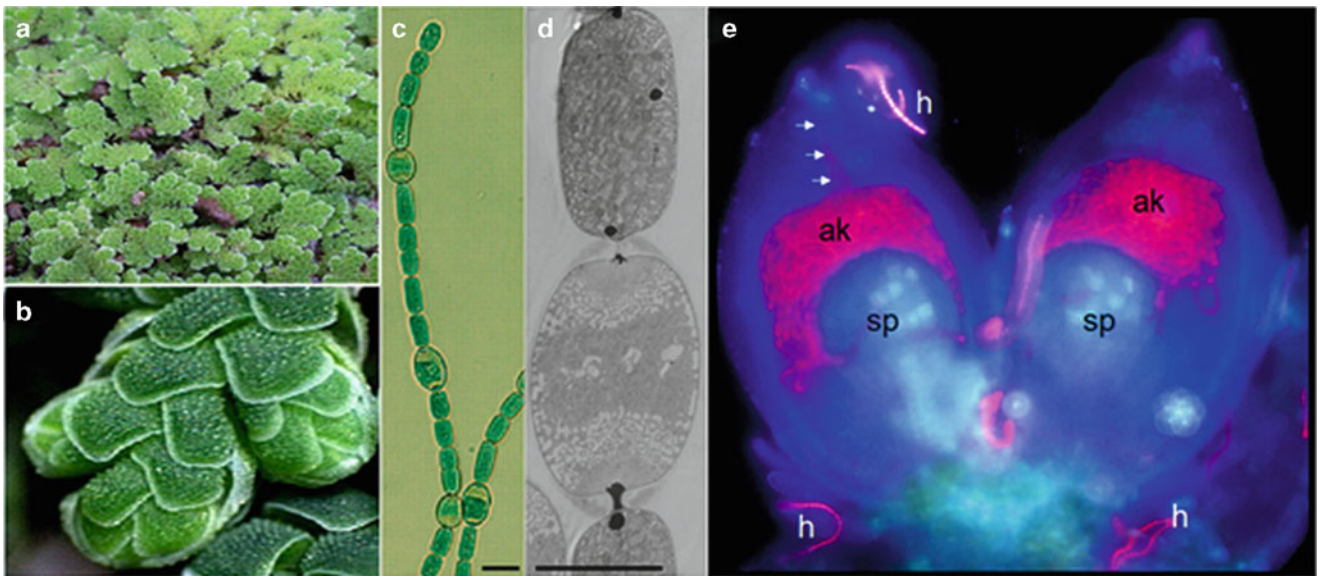

Fig. 23.7 The water fern *Azolla*. (**a**) View from above of *Azolla filliculoides* floating on the water surface. (**b**) View of an *Azolla* branch showing the overlapping dorsal lobes of the leaves which contain the cyanobionts. (**c**) Light micrograph of the *Azolla* cyanobiont with the large heterocysts clearly visible. (**d**) Transmission electron micrograph of a thin, longitudinal section of a cyanobiont filament showing a heterocyst (centre) with a vegetative cell on either side. (**e**) Fluorescence micrograph of a pair of megasporocarps (blue) which become infected with cyanobacteria when the motile hormogonia (*h*) on the surface, enter via channels (arrows). Once inside the megasporocarp the cells of the hormogonia convert into akinetes (ak) which can be seen as the intensely fluorescing area above the megaspores (sp). These akinetes provide the inoculum for the next generation of the fern, so maintaining the continuity of the symbiosis. Bars 5 μm (**c**), 5 μm (**d**). (Reproduced with permission from Ran et al. 2010)

23.2.1.5 Pteridophytes (*Azolla*)

Azolla consists of small (generally no greater than 3–4 cm) triangular or polygonal-shaped free-floating water ferns (Fig. 23.7a, b; Lechno-Yossef and Nierzwicki-Bauer 2002; van Hove and Lejeune 2002a, b; Bergman et al. 2007a, b). The genus *Azolla* comprises six or seven extant species traditionally grouped into two Sections, Azolla (synonymous with Euazolla) and Rhizosperma (Pabby et al. 2004a; Reid et al. 2006; Metzgar et al. 2007; Papaefthimiou et al. 2008b), primarily based on the characteristics of the reproductive structures. They are found worldwide from temperate to tropical climates on the surface of still or slow-moving bodies of freshwater such as ponds, paddy fields, ditches and marshes. *Azolla* coexists in mutual association with heterocyst-forming diazotrophic cyanobacteria and other eubacteria which are found in a cavity in the upper lobe of each leaf (Fig. 23.7). The *Azolla-Anabaena* symbiosis is the only example of a hereditary plant-cyanobacterial association in which the cyanobiont is transferred from one generation to the next. *Azolla* has wide agronomic and environmental applications including use as a green manure in rice cultivation (Vaishampayan et al. 2001; Bergman et al. 2007a, b), as a supplemental animal feed, in mosquito control, and in more recent years its potential for the removal of heavy metals from industrial effluent has been explored (van Hove and Lejeune 2002a, b; Choudhury and Kennedy 2004; Bennicelli et al. 2004; Tel-Or and Forni 2011). *Azolla*'s propensity for rapid growth also carries a disadvantage in some parts of the world where the plant is often regarded as a weed (Pabby et al. 2004b; Hashemloian and Azimi 2009).

Azolla has been used for centuries as a biological fertiliser in rice agriculture in China and other Far East countries and it has also been applied to enhance other crops, including bananas, wheat, tomato and taro (see: Vaishampayan et al. 2001; van Hove and Lejeune 2002a, b; Pabby et al. 2004a; Choudhury and Kennedy 2004; Franche et al. 2009). As well as nitrogen, *Azolla* also enriches soil fertility by supplying organic carbon, phosphorus and potassium (Pabby et al. 2004b). However, use of the crop has declined to less than 2% of the world's rice production, perhaps because its use is labour-intensive and the plant is susceptible to insect attack as well as being relatively sensitive to extremes of temperature and light intensity (Vaishampayan et al. 2001; Pabby et al. 2004b).

23.2.2 Fungi

23.2.2.1 Lichens

Lichens are stable symbiotic associations between a fungus (the mycobiont, which is usually an ascomycete) and a photosynthetic partner (the photobiont), which is a green alga or

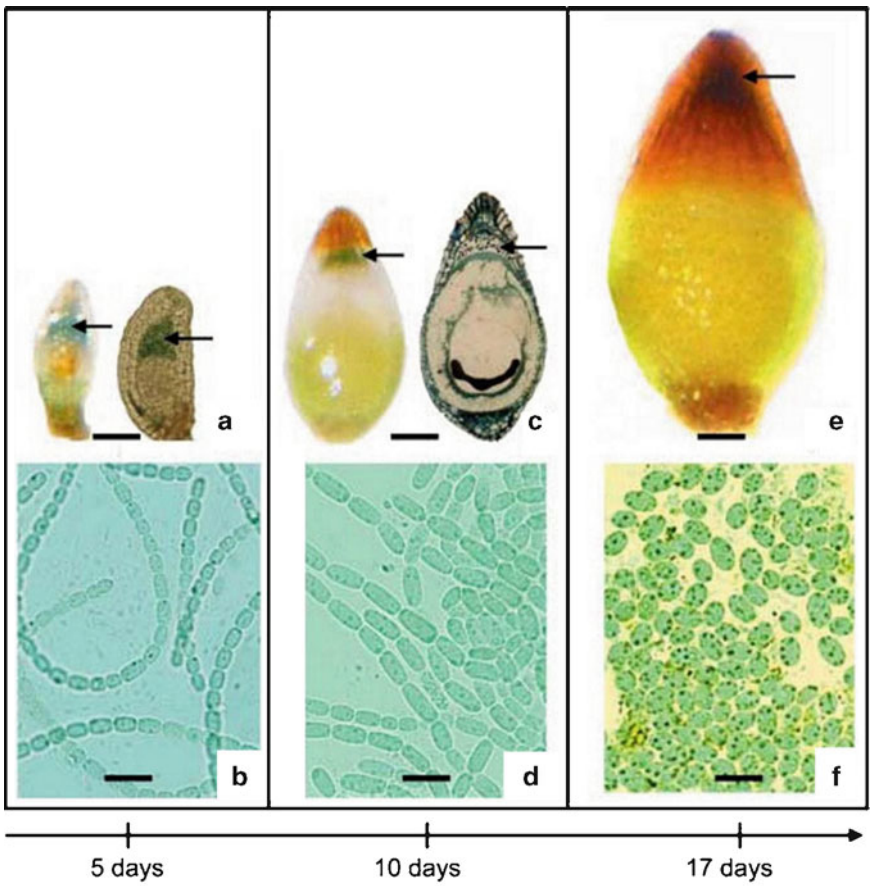

Fig. 23.8 Developmental sequence of the *Azolla microphylla* megasporocarp and its cyanobacterial colony. (**a**), (**c**) and (**e**) show a developmental sequence of the megasporocarp containing a cyanobacterial colony (arrow) in the indusium chamber above the megaspore. In (**a**) and (**c**) the intact megasporocarp is shown to the left and a semi-thin section to the right. (**b**), (**d**) and (**f**) show the morphology of the cyanobacteria in the megasporocarp represented by the developmental sequence shown in (**a**), (**c**) and (**e**) respectively. At 5 days (**b**) the cyanobacteria are mostly in the form of non-heterocystous hormogonial filaments. By 10 days (**d**) most cells have converted to large, elongated pro-akinetes. By 17 days (**f**) cells have developed into mature akinetes (a form of spore) containing numerous cyanophycin (nitrogen storage) granules. Bars 100 μm (**a**, **c**, **e**); 10 μm (**b**, **d**, **f**). (Reproduced with permission from Zheng et al. 2009b)

a cyanobacterium (Figs. 23.9 and 23.10; Sanders 2001, 2006; Rikkinen 2002; Rai and Bergman 2002; Oksanen 2006; Bergman et al. 2007a; Lücking 2008). Although not normally considered part of the symbiosis, bacterial communities growing as biofilms on the fungal surface may also be of importance (Grube et al. 2009), as may bacterial communities found within lichens (Bates et al. 2011). Of the 15,000–20,000 species of lichen, approximately 10% contain a cyanobacterium as the sole photobiont, and about 3% are so-called tripartite lichens which contain both a cyanobacterium (as the minor photobiont) and a green alga as the major photobiont (Rikkinen 2002; DePriest 2004; Adams et al. 2006; Bergman et al. 2007a). Of the nearly 20% of all fungi that form lichens, the vast majority are ascomycetes. Most cyanobionts are from the genus *Nostoc*, although members of other heterocystous and even unicellular genera are also involved (Rikkinen 2002). New cyanolichens and new cyanobionts are still being identified (Schultz et al 2000; Bjerke et al. 2003a; Grube 2005; Casamatta et al. 2006; Lücking et al. 2009) and this will no doubt continue. Some so-called cyanotrophic green algal lichens form either facultative or obligate associations with free-living N_2 fixing cyanobacteria, usually *Stigonema* or *Gloeocapsa*, presumably to access some of the N_2 they fix (Rikkinen 2002). Ascomycetes that obtain nutrients from cyanobacteria, often living within cyanobacterial colonies without forming a well-defined thallus, are also common although poorly understood (Rikkinen 2002).

Although the fossil record for lichens is poor (Rikkinen 2002), there is some evidence that lichen-like interactions between fungi and cyanobacteria or algae may have occurred over 600 million years ago, possibly in a shallow marine environment, long before vascular plants began to colonise the land (Yuan et al. 2005). Fossils identified as lichens have

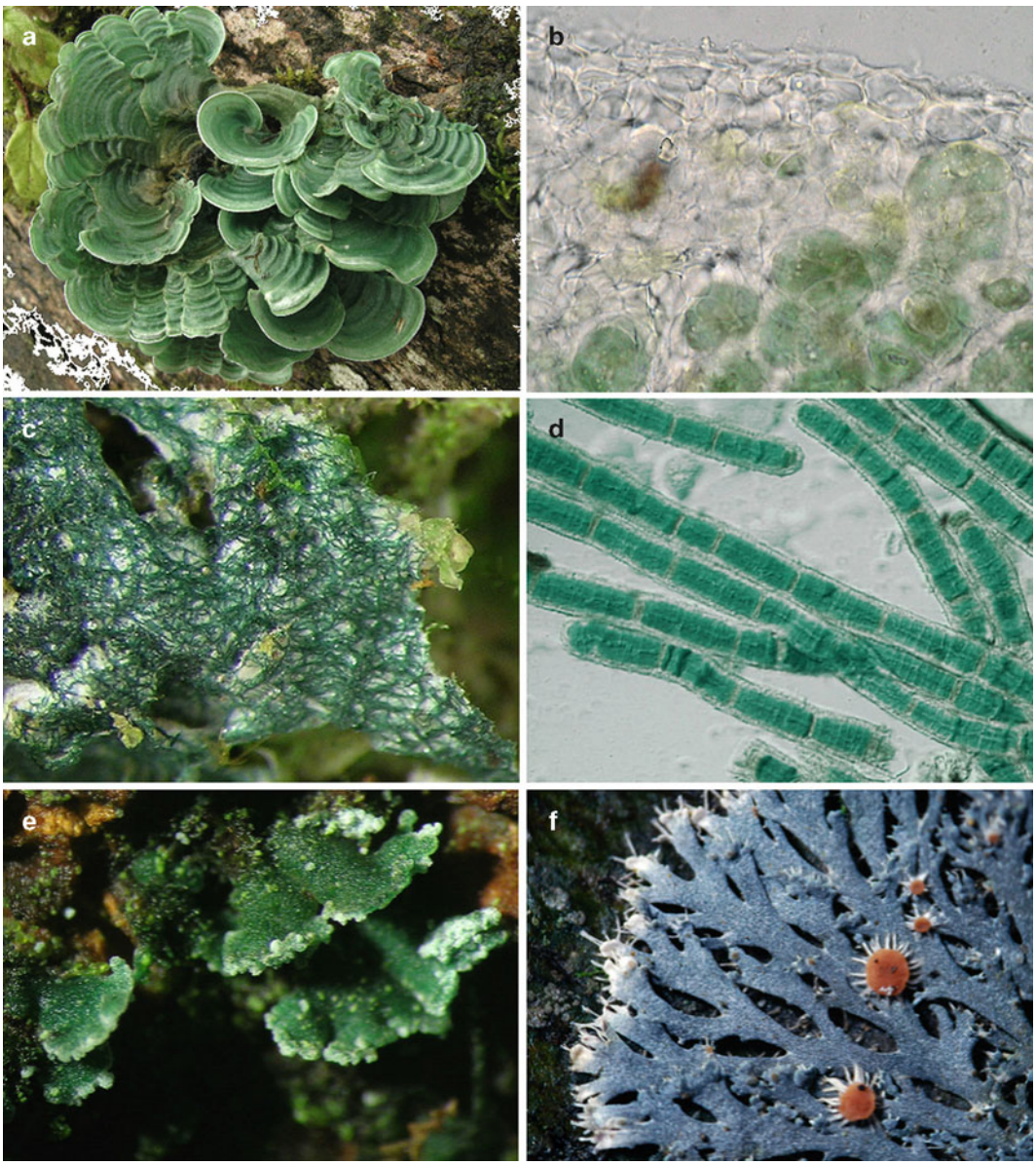

Fig. 23.9 Examples of tropical basidio- and ascolichens associated with a novel lineage of cyanobacterial photobionts. (**a**, **b**) *Dictyonema glabratum* foliose-lobate lichen thallus (**a**) and section through thallus showing cortex and globose photobiont cells (**b**). (**c**, **d**) *Dictyonema schenkianum* appressed-filamentous lichen thallus (**c**) and microscopic view of photobiont filaments surrounded by mycobiont hyphae (**d**). (**e**) *Acantholichen pannarioides* squamulose lichen thallus. (**f**) *Coccocarpia stellata* foliose lichen thallus. (Reproduced with permission from Lücking et al. 2009)

been found in 400 million year old rocks and in much younger amber (Rikkinen and Poinar 2002, 2008; Taylor and Krings 2005). The fossil lichen *Winfrenatia reticulata*, found in 400-million year old Devonian Rhynie chert in Scotland, is thought to have been a primitive form, consisting of a mycobiont and two cyanobionts, which has no analogue in extant lichens (Karatygin et al. 2009). The thallus consists primarily of dead and living filamentous cyanobacteria, implying that the fungus parasitized a cyanobacterial mat. It is clear from molecular studies that lichen symbioses have evolved independently on many occasions and some presently non-lichen-forming fungi may have evolved from ancient lichen-forming ancestors (Lutzoni et al. 2001; Rikkinen 2002).

Desiccation Tolerance

Many lichens experience a daily cycle of drying and wetting, and whilst in the desiccated state their photosynthetic apparatus is protected from potentially damaging levels of radiation both by a sunshade effect resulting from structural changes in the thallus as it dehydrates and by the production of a fluorescence quencher (MacKenzie and Campbell 2001;

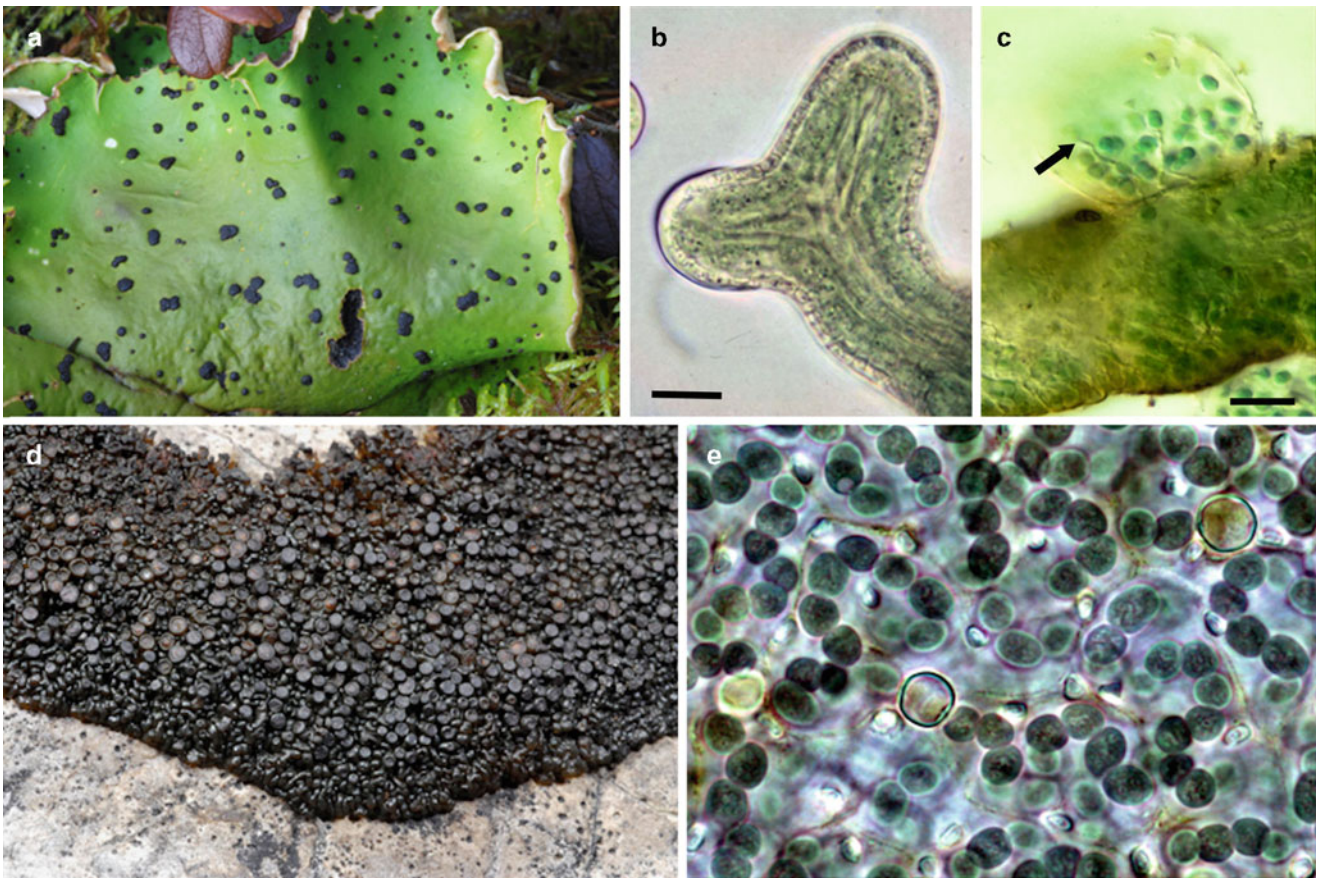

Fig. 23.10 Cyanolichens. (**a**) The tripartite cyanolichen *Peltigera aphthosa*. The green algal photobiont is visible over most of the thallus, whereas the cyanobiont is found in the brown cephalodia scattered across the surface. The white underlying fungal layer can be seen in places around the thallus periphery. (**b**) Lichen *Polychidium* sp. consisting of a single layer of fungal cells forming the cortex which surrounds the cyanobiont, *Scytonema*. (**c**) In the small, fruticose lichen *Lichinella stipatula* branching of the thallus first involves lateral emergence of the cyanobacterial symbiont (probably *Chroococcidiopsis* or *Myxosarcina*), as can be seen in this micrograph. Fungal hyphae (one of which is indicated by the arrow) then grow into the sheath material of the cyanobiont. (**d**) Thallus of the jelly lichen *Collema polycarpon* which has a brown-pigmented *Nostoc* cyanobiont. (**e**) The *Nostoc* cyanobiont of the lichen *Leptogium azureum* photographed through the upper cortex of the thallus. Large, thick-walled heterocysts can be seen amongst the more darkly pigmented vegetative cells. Bars 20 μm (**b**), 80 μm (**c**). (**b**) and (**c**) Reproduced with permission from Sanders WB (2006); (**a**), (**d**) and (**e**) courtesy of Jouko Rikkinen, University of Helsinki

Veerman et al. 2007). Recovery of photosynthesis following dehydration can be rapid in both cyanobacterial and green algal photobionts, although the former always require the presence of liquid water for recovery, whereas the latter can show significant recovery with elevated humidity alone (Palmqvist 2000; Kappen 2000; Lange et al. 2001). This difference is apparent in a lichen photosymbiodeme thallus (Sect. 23.5.5) in which the green algal section recovers photosynthetic activity at high humidity, whereas the cyanobacterial section only does so after rainfall (Schlensog et al. 2000; Green et al. 2002). The domination of green algal over cyanobacterial lichens in habitats such as humid, temperate, evergreen rainforests in New Zealand, northwest United States and Chile may therefore be a consequence of the frequent reactivation of photosynthesis in algal symbionts by humidity alone (Lange et al. 2001). By contrast cyanolichen photosynthesis is often severely limited by water availability. For example, in the lower montane tropical rain forests of Panama, where the tripartite cyanolichens *Lobaria crenulata*, *L. dissecta* and *Pseudocyphellaria aurata* and the bipartite cyanolichens *P. intricata*, *Stricta sublimbata* and *S. weigelii* are highly abundant, the water content of thalli is most favourable for photosynthetic activity at times of low light which limits such activity (Lange et al. 2004). Indeed, at optimum light intensities around noon, thalli are dry and so photosynthesis ceases. Cyanolichens are therefore favoured in moister woodland, or in microhabitats where moss cover or older tree bark provide a wetter environment (Ellis and Coppins 2006).

Desiccation also affects lichen N_2 fixation; in general, the longer the period of desiccation, the longer it takes for full recovery of N_2 fixation, although recovery is rapid in some lichens (Kranner et al. 2008). Another environmental factor that might influence cyanolichen N_2 fixation, particularly in polar regions, is increased UV radiation resulting from ozone depletion (Björn 2007). Indeed, field studies at a subarctic site on Svalbard have shown a 50% reduction in N_2 fixation

by the cyanolichen *Peltigera aphthosa* following 8 years of exposure to artificially-elevated UV-B radiation (Solheim et al. 2002). This appears to be a long-term effect as no reduction was seen after 11 weeks of exposure. *P. aphthosa* has a green algal primary photobiont and the cyanobacteria are found in external cephalodia on the surface of the thallus, fully exposed to UV-B radiation. By contrast, the cyanobacteria in the bipartite lichen *Peltigera didactyla* are protected by the overlying cortex and this may explain why, in similar field experiments, enhanced UV-B radiation had no effect on N_2 fixation in this cyanolichen (Bjerke et al. 2003b).

Habitats

Although a few cyanolichens live in marine littoral waters (Carpenter and Foster 2002), lichens are typically found in most terrestrial ecosystems and can become dominant in areas where their capacity to survive extremes of temperature and desiccation, and their ability to scavenge N or in the case of the cyanolichens fix their own N_2 gives them an advantage over vascular plants (Kappen 2000; Palmqvist 2002; Kranner et al. 2008). Although lichens grow very slowly, some cyanolichens can double their biomass in a year and can make significant contributions to the N budget of specific ecosystems such as montane forests (Büdel et al. 2000; Brown and Dalton 2002; Matzek and Vitousek 2003; Antoine 2004; Campbell and Fredeen 2007; Cusack et al. 2009; Menge and Hedin 2009) and biological soil crusts in arid regions such as the Colorado Plateau of North America (Belnap 2001, 2002) and southwestern Africa (Büdel et al. 2009). In the Colorado Plateau and other dryland regions cyanolichens such as *Collema tenax* and *Collema coccophorum* can be of major importance as part of biological soil crusts, contributing to erosion resistance and to regeneration following ecosystem damage (Bowker et al. 2010). Cyanolichens can also be important colonisers of bare rock such as recent lava flows (Crews et al. 2001; Kurina and Vitousek 2001).

In Antarctica cyanolichens are limited to the maritime regions, possibly because of the availability of the liquid water (from rainfall and meltwater) that they need for recovery of photosynthesis following desiccation (Kappen 2000). In arctic and subarctic regions N inputs from atmospheric deposition are low and the contribution of cyanolichens to the local N economy is significant; this contribution becomes even more important in the more extreme regions where N_2 fixing plants are rare (Weiss et al. 2005; Hobara et al. 2006). Indeed, if vascular plant abundance increases due to global warming this may result in macrolichen decline, as a result of the increased shading by the taller vascular plants and the litter they produce (Cornelissen et al. 2001; Weiss et al. 2005). In Arctic tundra, N_2 fixing lichens such as *Peltigera aphthosa* and *P. polydactyla* seem to be limited by phosphorus availability, because P-fertilisation can stimulate N_2 fixation and increase lichen nitrogen concentration (Weiss et al. 2005). By contrast, lichen abundance decreases significantly with ammonium nitrate fertilisation, perhaps as a result of increased shading by vascular plants (Weiss et al. 2005).

Cyanolichens are a particularly important part of the epiphyte community in the forests of the northern hemisphere, their prevalence increasing with the age of a forest and indeed, they are often restricted to old-growth forests (Sillett et al. 2000; Peterson and McCune 2001; Hedenås and Ericson 2004) although there are exceptions (Peterson and McCune 2003; Menge and Hedin 2009). Limitations in dispersal ability and diaspore production may be factors that restrict cyanolichens largely to old-growth forests (Hilmo 2002). Whereas lichens with green algal symbionts are ubiquitous, cyanolichens prevail in shady, humid stands in a boreal forest landscape (Hedenås and Ericson 2004), or in microhabitats where moss cover or older tree bark provide wetter conditions (Ellis and Coppins 2006). In addition, in these same forests the occurrence of cyanolichens correlates with the occurrence of their free-living cyanobionts, particularly on the shady, northern side of the tree trunks (Hedenås et al. 2007). Similarly, cyanolichen species richness and biomass are greatest in the more shady and humid parts of montane rainforests in Panama, where almost half of all lichen species are cyanolichens (Büdel et al. 2000).

In general, the factors that influence the occurrence and diversity of cyanolichens in a forest are, in decreasing order of importance, air quality, climate, elevation, soil nutrient status, forest age, proximity to deciduous trees, soil moisture and stand spacing (Goward and Arsenault 2000a, b). In some montane forests phosphate availability may constrain cyanolichen abundance as P-fertilisation can result in stimulation of the whole epiphyte community, but especially the cyanolichens (Benner and Vitousek 2007; Benner et al. 2007; McCune and Caldwell 2009). Where acid rain is prevalent cyanolichens are restricted to well-buffered bark, such as that of *Fraxinus* (ash), and can be lost from trees, such as *Quercus* (oak) and conifers, with more acidic bark (Richardson and Cameron 2004). This is a problem in much of Europe, but not in the west of Canada, nor in the American Pacific Northwest (Goward and Arsenault 2000a, b). In young forests of humid south-central British Columbia epiphytic cyanolichens, including the tripartite *Lobaria pulmonaria*, grow on the lower branches of conifers where calcium-rich leachates from adjacent *Populus* trees help to increase the pH of the conifer bark and encourage the initial establishment of epiphytic cyanolichens, although once they are established the presence of *Populus* is no longer essential (Goward and Arsenault 2000a). In forests on the border of Idaho cyanolichens only occur on the conifer branches that have low Mn/Ca and Mn/Mg ratios, which occurs within the drip zones of *Populus* trees (Hauck and Spribille 2002), implying that the ratio of minerals is more important than their concentration.

The tripartite cyanolichen *Lobaria pulmonaria* is still widespread in the northern hemisphere, but its abundance has decreased due to air pollution and forest management practises

(Gu et al. 2001). Factors affecting the spread of this lichen are the availability of suitable trees (notably aspen and willow) and the proximity of lichen-occupied trees (Gu et al. 2001). Although dispersal capacity may be a factor in limiting *L. pulmonaria* distribution (Öckinger et al. 2005), some consider ecological factors more important (Werth et al. 2006). Transplantation experiments have also shown that *L. pulmonaria* seldom achieves its growth potential in its natural ecological niches where there is a trade-off between optimum light intensity and the risk of desiccation damage, both of which occur higher in the tree canopy (Gauslaa et al. 2006). In deciduous forests cyanolichens such as *Lobaria pulmonaria* have to adapt to large fluctuations in light availability from low light in the summer when they are shaded by the tree canopy, to much higher light levels in winter and spring (MacKenzie et al. 2001). The best times for growth are the transition periods in spring and autumn when temperatures are relatively high, but the light is also at its greatest without the tree canopy. Both the intensity and the spectral quality of light available to lichens are also affected by the tree species and this in turn can result in chromatic adaptation in lichen cyanobionts and photobionts (Czeczuga et al. 2006, 2010).

Cyanolichens form a relatively small proportion of total forest biomass, but their abundance and N-rich thalli mean that they make substantial contributions to forest N particularly in old-growth forests which are commonly N-deficient (Campbell and Fredeen 2007; Botting et al. 2008). Nitrogen is released either by leaching from the lichen thallus or by decomposition of lichen litter (Holub and Lajtha 2003; Caldiz et al. 2007; Cornelissen et al. 2007). The thalli of bipartite cyanolichens generally have the highest N content, tripartite lichens lower and bipartite chlorolichens the lowest (Palmqvist et al. 2002; Caldiz et al. 2007; Botting et al. 2008). In addition, bipartite cyanolichens often show the most rapid rates of decomposition (Caldiz et al. 2007).

In forests, atmospheric deposition and litter decomposition can be the major contributors to the N budget, but in some northern forests, such as those in the American Pacific Northwest, atmospheric deposition is much less and the cooler temperatures, combined with the mainly lignin-rich coniferous litter, limit the rate of decomposition of soil organic matter, with the result that the contribution of cyanolichens is of greater significance, especially in old-growth or late-successional forests (Holub and Lajtha 2004; Knowles et al. 2006). For example, increases in soil N content can be measured up to 1.5 m away from thalli of terricolous cyanolichens of the genus *Peltigera* (Knowles et al. 2006) and epiphytic cyanolichens such as *Lobaria* can contribute 2.5–4.5 kg N ha^{-1} $year^{-1}$, which represents 33–67% of new N inputs into the local ecosystem (Holub and Lajtha 2004).

In addition to N_2 fixed by their cyanobionts, cyanolichens can obtain inorganic nitrogen such as nitrate and ammonium, and organic forms such as amino acids, from rainwater and canopy through-fall. Indeed, these forms of N are taken up by cyanolichens, although there seems to be little correlation between rates of uptake and lichen morphology or microhabitat (Dahlman et al. 2004). Despite large variations in N supply cyanolichens are able to maintain a steady thallus N content and to regulate the distribution of nitrogen and carbon resources around the thallus (Sundberg et al. 2001; Dahlman et al. 2002, 2004; Kytöviita and Crittenden 2007).

In addition to the more obvious environmental influences described above, forest management practices can have significant effects on cyanolichen survival and abundance. With the fragmented nature of much woodland in Europe, existing patches of old-growth woods may be vital for the preservation of lichens in times of environmental stress such as global warming (Ellis et al. 2009). The aspen (*Populus tremula*) is a common host of epiphytic cyanolichens in Sweden, but new stands of aspen are often not colonised for 50 or more years, so loss of trees or changes to the forest structure or composition, can lead to a long-term decline in cyanolichen abundance (Hedenås and Ericson 2004), and careful management of forests is vital for the preservation of lichen populations (Hedenås and Ericson 2003; Richardson and Cameron 2004; Hedenås and Hedström 2007). Cyanolichens are most common on aspen stands within coniferous forests, and are rare on aspen growing in previously agricultural land, possibly because they are at an advantage in the forest where N availability is low (Hedenås and Ericson 2004). However, not all cyanolichens respond in the same way to changes in forestry practices. For example, of three foliose cyanolichens in the Collemataceae, *Collema curtisporum* and *C. furfuraceum* are 5–6 times less frequent in aspen-poor coniferous forest than in aspen-rich coniferous forest, whereas *Leptogium saturninum* is unaffected by aspen abundance, perhaps because it has better dispersal abilities (Hedenås and Ericson 2008). However, even following dispersal the juvenile stages of epiphytic cyanolichens are sensitive to environmental factors which may result in very low growth and colonization (Hilmo and Ott 2002).

A final major influence on lichen populations is likely to be climate change, but a study by Ellis and Coppins (2007) illustrates the difficulties of predicting the outcomes. They modelled a community of lichen epiphytes, consisting of 80% cyanolichens, and known as the *Lobarion*, named after *Lobaria pulmonaria*. This population, which is characteristic of the cool temperate forests of western Scotland and south-western Norway, is sensitive to climatic and habitat changes and could be favoured by predicted increases in average annual temperatures and winter precipitation, which might result in an increase in its current range (Ellis and Coppins 2007). However, modelling revealed a complex relationship between temperature, precipitation and woodland structure, such that the response of the *Lobarion* to climate change may be modified by the current, rather than the future forest landscape. In a recent study of lichens in Italy, Marini et al. (2011) concluded that the future impacts of climate change

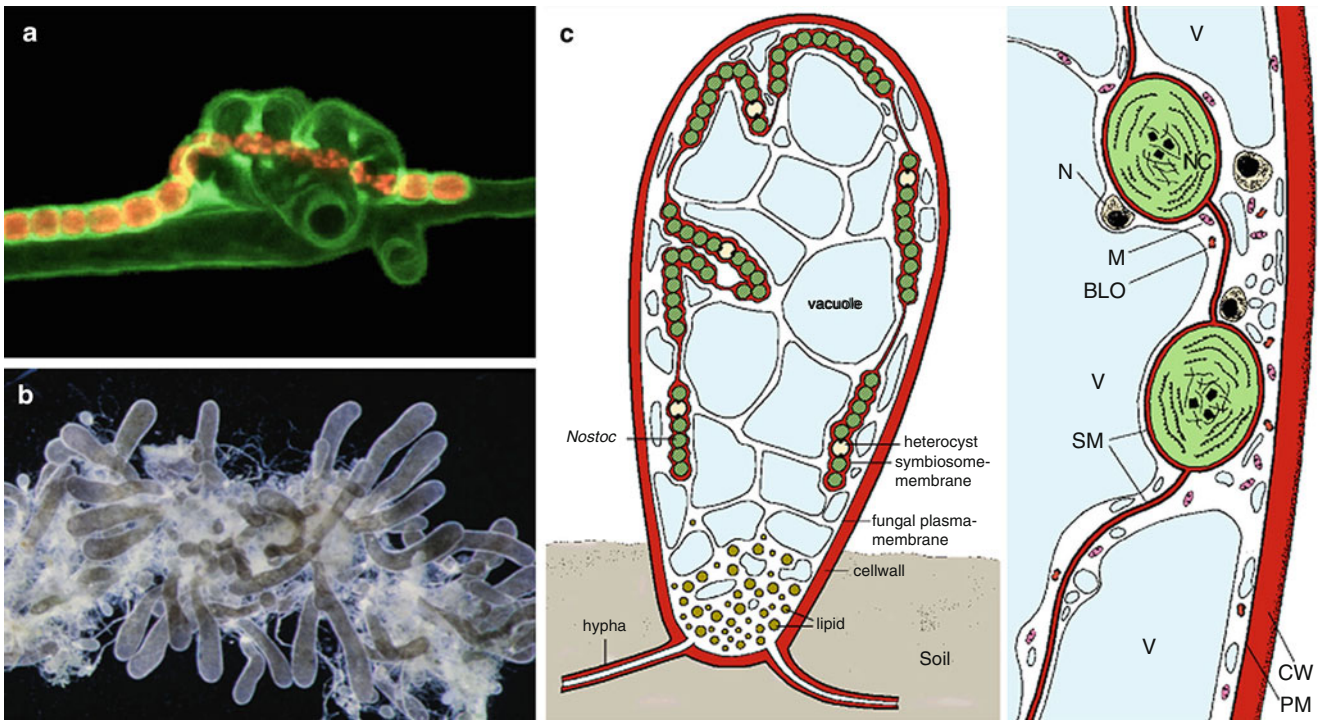

Fig. 23.11 The *Geosiphon-Nostoc* symbiosis. (**a**) Confocal laser scanning microscopic projection of a *Geosiphon* hypha engulfing a *Nostoc* filament (the cells of which are 3–5 μm in width). The fungal cell wall and the *Nostoc* extracellular polysaccharides have been labelled with the fluorescence-coupled lectin ConA (green). In the centre of the image the *Nostoc* cells (red) that have been engulfed show deformations and reduced pigment fluorescence, whereas those on either side retain their normal appearance. (**b**) The fully-developed symbiosis in which the cyanobiont is contained in bladders, the largest of which are about 1.5 mm in length. (**c**) Diagrammatic representation of the *Geosiphon-Nostoc* symbiosis, showing the compartmentation of the cyanobiont. The drawing to the right shows an enlargement of the peripheral region of the bladder. Drawings are based on electron microscope observations. *BLO* bacteria-like organism, *CW* cell wall, *M* mitochondrion, *N* nucleus, *NC Nostoc* cell, *PM* plasma membrane, *SM* symbiosome membrane, *V* vacuole. (Reproduced with permission from Adams et al. 2006)

on lichen species richness is likely to vary with photobiont (green algal or cyanobacterial) type.

Lichens as Food

Lichens form a vital part of the winter diet for reindeer and caribou (den Herder et al. 2003), yet these animals avoid eating cyanolichens, even when starving, despite them being a far richer source of N than green algal lichens (Rai 2002). This was thought to be because of the poor digestibility of some cyanolichen species (Storeheier et al. 2002), but there is an interesting alternative or additional explanation. The *Nostoc* symbionts of the cyanolichens *Pannaria pezizoides* and *Peltigera leucophlebia* have been shown to produce hepatotoxic microcystins, both in the cephalodia (in the case of the latter lichen) and when grown free-living in culture and it might be this cyanobacterial toxin that deters grazers (Oksanen et al. 2004; Kaasalainen et al. 2009). As Kaasalainen et al. (2009) have pointed out, the production of microcystin by lichen-associated *Nostoc* raises concerns about the safety of such lichens used in China as food and in traditional medicine. Although in many tripartite lichens it is the cephalodia that are avoided by grazers, the arctic tripartite cyanolichen *Nephroma arcticum* has a *Nostoc* photobiont in internal cephalodia which are preferentially grazed by slugs, while the green algal parts of the thallus are mostly left alone (Asplund and Gauslaa 2010).

23.2.2.2 *Geosiphon pyriformis*

The *Geosiphon pyriformis-Nostoc* symbiosis (referred to as *Geosiphon pyriforme* in older literature) is the only known example of an endocytobiotic cyanobacterium-fungus association (Kluge et al. 2002; Schüßler 2002; Adams et al. 2006; Bergman et al. 2007a). The fungal host belongs to the arbuscal mycorrhizal (AM) and related fungi within the phylum *Glomeromycota* (Schüßler et al. 2001; Kluge 2002; Adams et al. 2006). The cyanobiont, *Nostoc punctiforme*, is found intracellularly within specialised bladders produced by the fungal hyphae (Fig. 23.11). Although the cyanobacterium can be grown free of the fungus, the fungus appears to be an obligate symbiont (Schüßler 2006). The function of the *Nostoc* seems to be primarily the provision of photosynthate for the fungus, although the presence of heterocysts and high nitrogenase activity within bladders clearly indicate that N_2 fixation also occurs (Adams et al. 2006). The *Nostoc*-containing bladders can only take up molecules with a diameter less than

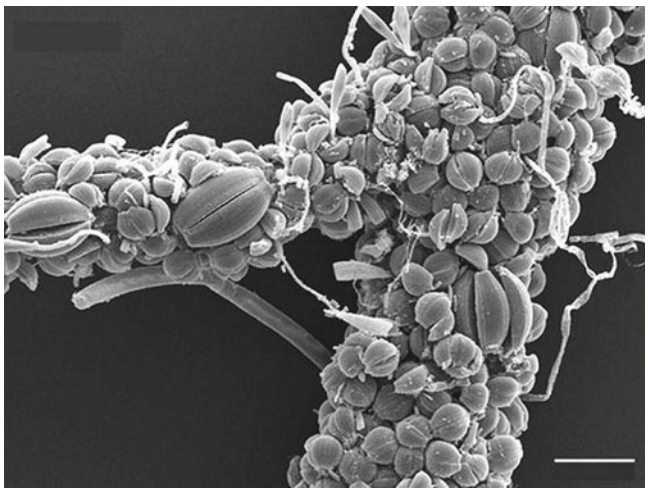

Fig. 23.12 Scanning electron micrograph of the filamentous freshwater green alga *Cladophora* covered with epiphytic diatoms. The larger diatoms are *Epithemia turgida* and the smaller ones are *E. sorex*, both of which contain N_2-fixing cyanobacterial endosymbionts. Bar 50 µm. (Photograph by Rex Lowe. Reproduced with permission from Power et al. 2009)

0.45 nm, which excludes sugars but not inorganic ions such as phosphate which can therefore be supplied to the cyanobiont. Indeed, phosphate limitation is a strong promoter of the formation of the association (Adams et al. 2006) (Fig. 23.12).

The *Geosiphon pyriformis-Nostoc* symbiosis is found in the upper layers and on the surface of moist, nutrient-poor soils, particularly those low in phosphate. It seems to be rare, there having been only five reports of its occurrence in nature, at sites from Eastern Germany to Austria. Although it can be grown successfully in the laboratory it is difficult to obtain large amounts of material for experimentation (Kluge et al. 2002; Adams et al. 2006). In nature the *Nostoc* cyanobiont is thought to be released from decaying *Geosiphon* bladders in the form of akinetes which subsequently germinate. There is evidence that the same *Nostoc* symbionts can be shared by *Geosiphon pyriformis*, the liverwort *Blasia* and the hornwort *Anthoceros*, which are all found in close proximity in their natural environment (Adams et al. 2006).

23.2.3 Diatoms

A number of mostly marine diatom-cyanobacteria symbioses are known and there are surely more to be discovered, particularly as the cyanobiont is often difficult to visualise by microscopy. The heterocystous cyanobacterium *Richelia intracellularis* (Hindák 2000), which consists of a short filament of 3–10 vegetative cells with a single heterocyst at one end, is found as an endosymbiont in diatoms of genera *Rhizosolenia* and *Hemiaulus* which are abundant in tropical and sub-tropical seas (Fig. 23.13c; Bergman 2001; Janson 2002; Carpenter 2002; White et al. 2007; Bar Zeev et al. 2008; Foster et al. 2007, 2009; Wouters et al. 2009; Bombar et al. 2011). *R. intracellularis* has also been found as an epiphyte on the diatom *Chaetoceros compressus* (Fig. 23.13a, b) in the Pacific and Indian Oceans (Gómez et al. 2005) and in the Atlantic Ocean (Foster et al. 2009). The epiphyte of *Chaetoceros* has sometimes been referred to as *Calothrix rhizosoleniae*, although Foster and Zehr (2006) have shown it to be closely related to, but distinct from, the *Richelia* endophytes from *Hemiaulus hauckii* from the North Atlantic and *Rhizosolenia clevei* from the North Pacific. These N_2 fixing *Richelia*-diatom symbioses may make major contributions to the N budgets of the areas of ocean where they are abundant, especially when they form blooms, some of which can cover areas of over 100,000 km^2 (Zehr et al. 2000; Arrigo 2005; Mahaffey et al. 2005; White et al. 2007; Foster et al. 2009). Foster et al. (2011) have recently demonstrated the mutualistic nature of these diatom-cyanobacteria symbioses. *Richelia* was show to fix far more N_2 than was needed for its own use, up to 97.3% of this N being transferred to the host. In turn, rates of both N_2 fixation and growth of the *Richelia* symbionts were much greater in symbiosis than in the free-living state.

Unicellular cyanobacteria are also found as symbionts of diatoms. For example, the chain-forming diatoms *Neostreptotheca* and *Streptotheca*, which are common in the tropics, house numerous 3–5 µm diameter cyanobacterial cells in their cytoplasm (Carpenter 2002). Each cell of another chain-forming diatom, *Climacodium frauenfeldianum*, contains 20–30 coccoid cyanobacteria, thought to be related to the N_2 fixing genus *Cyanothece* (Carpenter and Janson 2000; Carpenter 2002). The cytoplasm of diatoms *Rhopalodia gibba* and *Epithemia turgida* contains two to five unicellular endosymbionts known as spheroid bodies, which are clearly related to cyanobacteria yet appear to have lost the capacity for photosynthesis (Janson 2002; Prechtl et al. 2004; Kneip et al. 2008; Bothe et al. 2010). The *Rhopalodia/Epithemia*-cyanobacteria associations can fix N_2 in the light and phylogenetic analysis of cyanobiont 16S rDNA and *nifD* has revealed a close relationship to the N_2 fixing cyanobacterium *Cyanothece* (Prechtl et al. 2004; Bothe et al. 2010).

A freshwater example of a cyanobacterial-diatom symbiosis is found in the Eel River in California, U. S. A., where heavy growths of the macroalga *Cladophora* become overgrown by the diatoms *Epithemia turgida* and *Epithemia sorex*, and to a lesser extent *Rhopalodia gibba*, all with cyanobacterial endosymbionts (Fig. 23.12; Power et al. 2009). These epiphytic *Epithemia* biofilms can fix N_2 at rates ranging from 0.3 to 1.7 µg N g^{-1} (dry wt) h^{-1}. The great abundance of *Cladophora* increases the functional surface area of the littoral zone by a factor of up to 2×10^5 and when this surface is covered by N_2 fixing *Epithemia*, the contribution to ecosystem N could be enormous (Power et al. 2009).

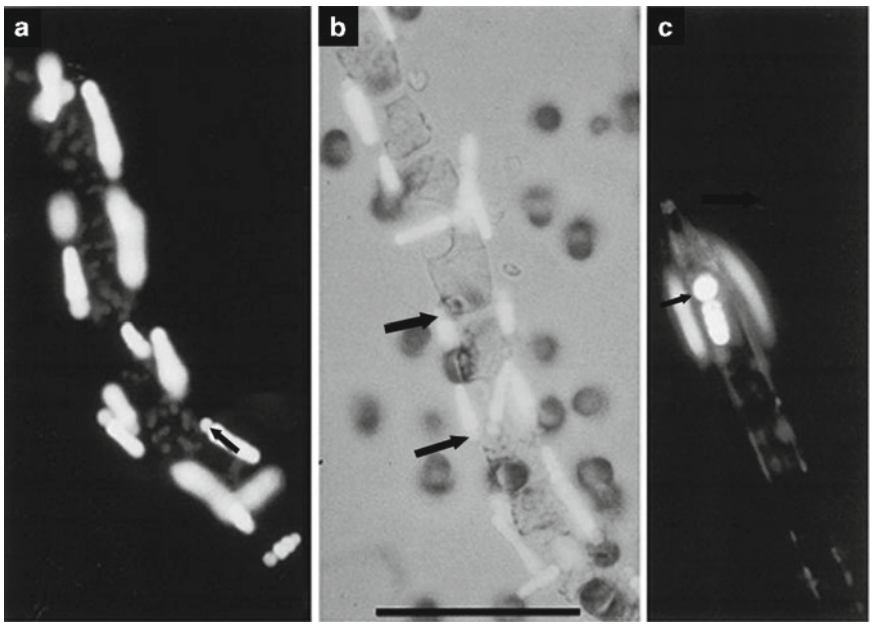

Fig. 23.13 Diatom symbioses with the cyanobacterium *Richelia intracellularis*. (**a**) Epifluorescence micrograph of fluorescing *R. intracellularis* filaments attached to the outside of the chain-forming diatom *Chaetoceros* with its more weakly fluorescing chloroplasts. The arrow indicates the single small heterocyst found at one end of each filament. (**b**) Merged fluorescence and incident light images of *Chaetoceros* with epibiotic *R. intracelluaris* filaments. The dark, roughly circular patches in the background are the pores of the filter used to concentrate the sample. (**c**) Single *R. intracellularis* filament inside *Rhizosolenia clevei* var. *communis*. The arrow indicates the single large heterocyst. Bar 50 μm. (Reproduced with permission from Janson et al. 1999)

A three-membered symbiosis is formed by the centric diatom *Leptocylindrus mediterraneus* which carries in its girdle bands the aplastidic protist *Solenicola setigera*, found as groups of cells together with abundant coccoid cyanobacteria, thought to be *Synechococcus*, embedded in the protist's extracellular matrix (Carpenter 2002). Although both the protist and the *Synechococcus* are widely-distributed, the three-membered symbiosis seems to be rare.

A potentially symbiotic relationship is formed by motile diatoms of the genera *Amphora*, *Berkeleya*, *Cymbella*, *Entomoneis*, *Epithemia*, *Lunella*, *Mastogloia*, *Nitzschia* and *Rhopalodia* which are found within colonies of the heterocystous cyanobacterium *Rivularia* Roth in the Baltic Sea (Snoeijs and Murasi 2004). This is thought to benefit the diatoms by protecting them from grazing and physical disturbance, providing mucilage as a substratum for motility and supplying nutrients released by the *Rivularia*, although the benefits to the cyanobacteria are unclear.

Another unusual interaction between cyanobacteria and diatoms is the formation of "microbial spheres" found in North Sea microbial mats (Brehm et al. 2003). These spheres are up to 3 mm in diameter and consist of a complex community of heterotrophic bacteria, diatoms (*Navicula perminuta*) and cyanobacteria (*Phormidium*) embedded in extracellular polymeric substances and surrounded by a membrane of unknown composition. The spheres can be maintained in laboratory culture for 3 years or more and can become calcified, producing ooids (Brehm et al. 2006). Similar microbial consortia have been found in a desert spring in Mexico (Garcia-Pichel et al. 2002). These 'waterwarts' are roughly spheroid or elongated colonies approximately 1 cm in diameter, consisting of *Aphanothece*-like unicellular cyanobacteria embedded in large amounts of gel-like glycan. These colonies support an assemblage of filamentous cyanobacteria (including possible *Phormidium*, *Pseudanabaena* and *Lyngbya*) and diatoms (primarily *Nitzschia*). The colonies also invariably contain crystals of calcite which are thought to act as ballast, preventing the colonies from being washed out of the spring by the upwelling water (Garcia-Pichel et al. 2002).

23.2.4 Dinoflagellates, Radiolarians, Tintinnids, Euglenoids and Foramenifera

Non-photosynthetic dinoflagellates (Dinophycaea) were shown to harbour epi- or endobiotic "phaeosomes" over a century ago (see: Carpenter 2002; Carpenter and Foster 2002; Foster et al. 2006a). These phaeosomes are now known to be symbiotic cyanobacteria, but for a long time little was known about them. Members of six dinoflagellate genera, *Ornithocercus*, *Histioneis*, *Parahistioneis*, *Citharistes*, *Dinophysis* and *Amphisolenia*, have been reported to contain a range of unicellular symbiotic cyanobacteria (Fig. 23.14; Carpenter 2002;

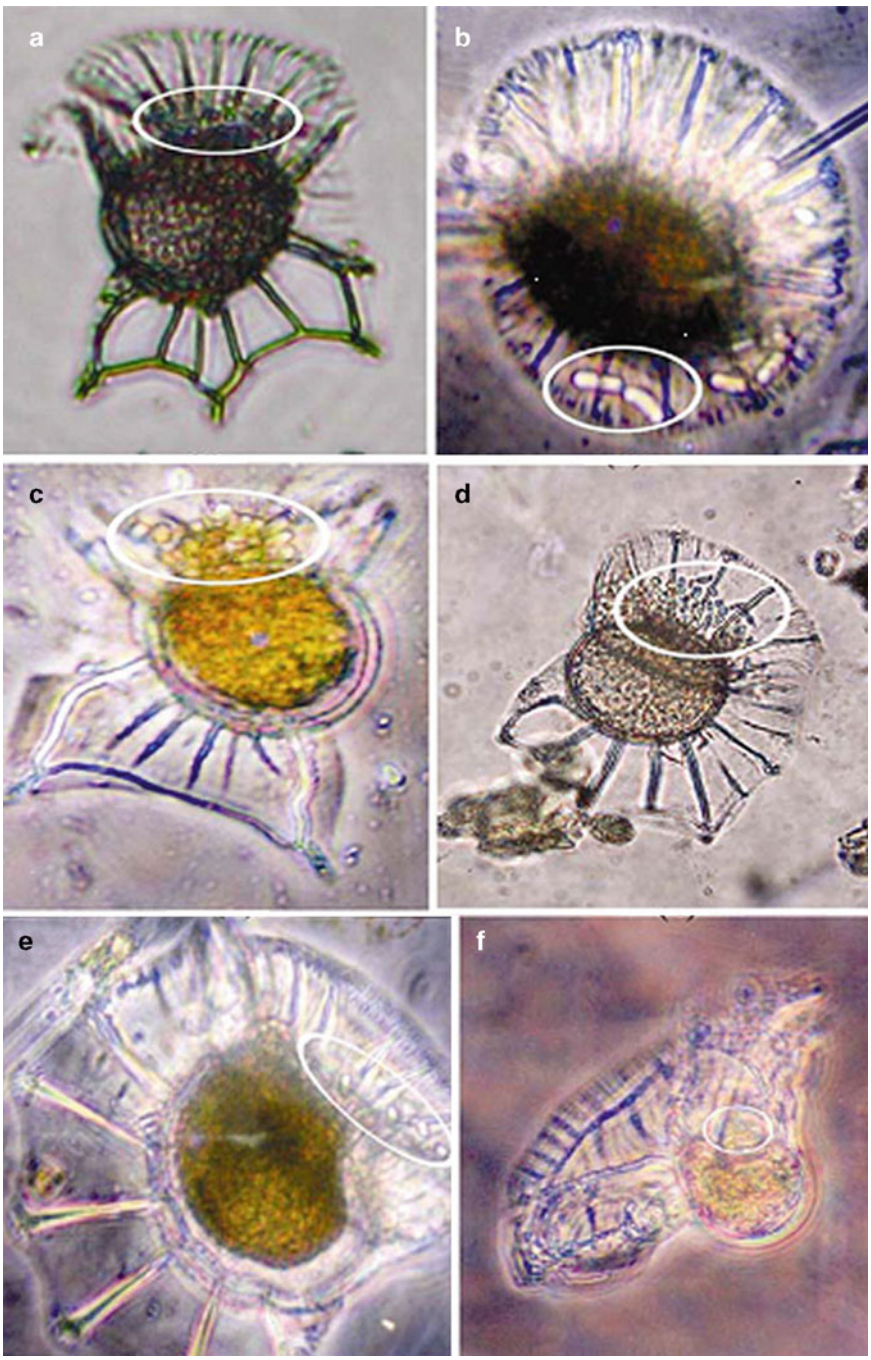

Fig. 23.14 Dinoflagellate-cyanobacteria symbioses. Heterotrophic dinoflagellates (**a**) *Ornithocercus magnificus*, (**b**) *O. quadratus*, (**c**) *O. heteroporus*, (**d**) *O. thumii*, (**e**) ***O****. steinii* and (**f**) *Histioneis hyaline* (**f**) with the location of the cyanobionts indicated by circles. (Reproduced with permission from Jyothibabu et al. 2006)

Jyothibabu et al. 2006; Tarangkoon et al. 2010), which in the case of *Histioneis* label positively with anti-nitrogenase antibodies, so may be capable of N_2 fixation (Foster et al. 2006a).

The radiolarians are amoeboid protozoa with mineral skeletons and at least two of them, *Spongostaurus* and *Dictyocoryne truncatum*, host symbionts thought to be related to *Prochlorococcus* (Foster et al. 2006a, b). Tintinnids are conical or trumpet-shaped protozoan ciliates, at least one of which, the open-ocean tintinnid *Codonella*, hosts cyanobacterial symbionts (Carpenter and Foster 2002; Foster et al. 2006a). An apparently transient and rare endosymbiosis is formed between the euglenoid *Petalomonas sphagnophila* and *Synechocystis*-like unicellular cyanobacteria (Schnepf et al. 2002). This apoplastidic euglenoid flagellate is found in floating mats of *Sphagnum* moss in bog lakes in Germany and the cyanobacteria were once thought to be food particles, but they remain alive for several weeks or longer inside a perialgal vacuole, even though digestion of food particles is usually complete within a few hours. The foraminifera (amoeboid protists) *Marginopora vertebralis* and *Amphisorus*

hemprichii, have also been reported to contain endophytic cyanobacteria (Lee 2006).

23.2.5 Animals

23.2.5.1 Sponges

Sponges (phylum *Porifera*) are some of the most ancient metazoan animals, with a fossil record dating back over 580 million years to the Precambrian (Taylor et al. 2007). Research on sponges has increased rapidly in the last decade or so, partly because of the interest in the complex populations of symbiotic microorganisms they host, but perhaps largely because they produce a wide array of biologically-active secondary metabolites, some of which may be produced by their symbionts, which include cyanobacteria (Lee et al. 2001; Flatt et al. 2005; Ridley et al. 2005a, b; Schmidt et al. 2005; Taylor et al. 2007; Kennedy et al. 2007, 2008; Simmons et al. 2008; Selvin et al. 2010; Sacristan-Soriano et al. 2011; Li et al. 2011). The symbiotic microorganisms in sponges can constitute up to 40% of the host biomass and can exceed the microbial concentration in the surrounding seawater by up to four orders of magnitude (Friedrich et al. 2001; Webster and Hill 2001; Hentschel et al. 2006; Taylor et al. 2007; Schmitt et al. 2008). The sponge microbial population can be highly diverse; for example, from 10 individuals of the sponge *Candidaspongia flabellate* Burja and Hill (2001) were able to isolate in culture 228 different bacterial species, 25 fungi, 3 actinomycetes and 9 cyanobacterial strains. In photosynthetic sponges the symbionts include eukaryotic rhodophytes, diatoms, dinoflagellates and chlorophytes, but the most important and abundant group is probably the cyanobacteria (Wulff 2006; Hentschel et al. 2006; Taylor et al. 2007; Usher 2008; Hardoim et al. 2009).

Of the sponges hosting cyanobacterial symbionts (cyanosponges) 100 species are known (Diaz et al. 2007; Usher 2008) and they typically constitute 30–50%, but sometimes up to 90%, of the sponges on tropical reefs (Usher 2008). They are thought to be the most ancient of the microorganism-metazoan interactions (Hentschel et al. 2006). Cyanosponges were once thought to be mostly restricted to the nutrient-poor water of tropical regions, but later reports suggest they are just as common in temperate waters (Usher 2008). Their colours usually result from the cyanobiont phycobiliproteins, the ratios of which can vary depending on the amount of light received, resulting in colour changes from yellow/green in high light to red/brown in low light (Usher et al. 2004a). Cyanobacterial pigments may also provide the sponge with protection from excessive sunlight, particularly in intertidal zones (Taylor et al. 2007). Sponge cyanobionts seem to substantially enhance host growth rates in at least two Caribbean coral reef sponges, *Aplysina fulva* and *Neopetrosia subtriangularis* (Erwin and Thacker 2008a). Indeed, cyanosponges in general seem to be faster growing and more competitive for space than the non-photosynthetic sponges and can even overgrow and kill live coral (Diaz et al. 2007; Usher 2008; Tang et al. 2011; Hirose and Murakami 2011). However, the degree of dependence in cyanobacteria-sponge associations may vary. For example, when artificially shaded the marine cyanosponges *Lamellodysidea chlorea* and *Xestospongia exigua* respond differently, the former losing mass while its cyanobiont (the filamentous *Oscillatoria spongeliae*) doesn't change in abundance, implying a mutualistic relationship, whereas the latter does not lose mass but its cyanobiont (the unicellular *Synechococcus spongiarum*) decreases in abundance, implying a commensal relationship (Thacker 2005). Similarly, bleaching of the *Synechococcus* symbionts of the giant barrel sponge *Xestospongia muta* in the Florida Keys does not result in sponge mortality (McMurray et al. 2011). By contrast, bleaching of the Mediterranean sponge *Ircinia fasciculata* results in the death of its symbiotic cyanobacteria and the subsequent death of the sponge itself (Cebrian et al. 2011).

Cyanosponges play important roles in reef ecology as nutrient cyclers and primary producers and they provide food and a habitat for a wide range of organisms (Usher 2008). Their cyanobacterial symbionts confer several advantages over the zooxanthellae in other photosynthetic sponges, because they have a wider temperature tolerance, produce sunscreens and can photosynthesise at very low light, enabling their hosts to grow in full sun in intertidal zones and at low light in shaded areas and even in caves (Usher 2008). Although digestion of the cyanobionts, as a potential food source, was reported in earlier studies, it is likely that this is rare and may be a result of poor health of one of the partners (Usher 2008). Cyanobiont secondary metabolites can provide the host with protection from grazing, although some cyanobacteria may actually attract predators to feed on sponges, as the mollusc *Tylodina perversa* chooses to feed on areas of *Aplysina aerophoba* rich in symbiotic cyanobacteria, but it shows no interest in the closely-related *Aplysina cavernicola* which lacks cyanobionts (Becerro et al. 2003).

23.2.5.2 Corals

The primary photosynthetic symbionts of scleractinian (stony) corals are a diverse group of endosymbiotic dinoflagellates (zooxanthellae) of the genus *Symbiodinium* which are found within the gastrodermal cells of the host (Knowlton and Rohwer 2003; Rosenberg et al. 2007; Chen et al. 2011). However, corals also harbour diverse bacterial communities (Rosenberg et al. 2007; Chen et al. 2011) and cyanobacterial symbionts are found in at least one coral, *Montastreae cavernosa* (Lesser et al. 2004, 2007). Cyanobacterial sequences have also been found by PCR amplification of total DNA from three coral species, *Montastraea franksi*, *Diploria strigosa* and *Porites astreoides* using bacteria-specific 16S rDNA primers (Rohwer et al. 2001, 2002). In addition, cyanobacterial DNA sequences have been identified in the metagenome

Fig. 23.15 Caribbean scleractinian (stony) coral *Montastraea cavernosa* showing orange daytime fluorescence from the phycoerythrin of its unicellular cyanobacterial endosymbionts. The N_2 fixing intracellular cyanobionts co-exist with zooxanthellae. The colony is approximately 0.6 m in height. (Reproduced with permission from Lesser et al. 2004)

of the bacterial community from the coral *Porites compressa* (Thurber et al. 2009) and the cyanobacterial *nifH* gene has been detected in *Montipora* spp. (Olson et al. 2009).

The characteristic sun-induced orange-red fluorescence of the Caribbean coral *Montastreae cavernosa* (Fig. 23.15) is derived from the red cyanobacterial photopigment phycoerythrin, found in 1.0–3.0 μm diameter unicellular cyanobacteria within the epithelial cells of the host and surrounded by host cell membrane (Lesser et al. 2004). The 16S ribosomal DNA sequence of these coccoid cyanobacteria is most closely related to either *Synechococcus* or *Prochlorococcus* in the order Chroococcales. The fluorescence is apparently caused by detachment of the phycoerythrin from the photosynthetic apparatus, induced by the presence of high concentrations of glycerol, which is the major carbon compound transferred from the zooxanthellae to the coral host and which may also act as a carbon source for the cyanobionts (Lesser et al. 2004). The cyanobacteria are capable of N_2 fixation, which is confined to the night when the host tissues revert from their daytime hyperoxia to the hypoxia or anoxia that favours N_2 fixation (Lesser et al. 2007). At least some of the N_2 fixed is found in the zooxanthellae. If N_2-fixing cyanobacteria prove to be widespread in corals, they (together with the many other N_2-fixing bacteria) may play a significant role in the N-budget of coral reefs (Lesser et al. 2007).

The calcareous skeleton of scleractinian corals can provide a home for endolithic cyanobacteria and algae. For example, the encrusting coral *Oculina patagonica*, with its endosymbiotic zooxanthellae harbours endolithic chlorophytes of the genus *Ostreobium* in its skeleton (Fine and Loya 2002). Transfer of the products of photosynthesis from the endolithic algae to the coral may help survival of the coral during periods of bleaching when loss of the symbiotic zooxanthellae allows more light to reach the endoliths (Fine and Loya 2002). The endolithic, filamentous cyanobacterium *Plectonema terebrans* can be found burrowing into the calcareous skeleton of the cold-water corals *Desmophyllum dianthus* and *Caryophyllia huinayensis*, which lack zooxanthellae (Försterra and Häussermann 2008). The cyanobacterium is visible as a pink to violet discolouration of the corallite (Fig. 23.16) and is frequently found together with the endolithic filamentous green alga *Ostreobium queckettii* (Fig. 23.16), with the cyanobacterium generally most abundant on the light-facing side of the corallite. Both endoliths are most abundant where the corallite is covered with polyp tissue, possibly as a result of protection from grazers. Excreted metabolites of the endolithic phototrophs may be beneficial to the host polyp although the nature of these metabolites is not known (Försterra and Häussermann 2008).

23.2.5.3 Ascidians

Ascidians or sea squirts are sac-like marine invertebrate filter feeders, approximately 30 of which, from four genera of the Didemnidae (*Didemnum*, *Trididemnum*, *Lissoclinum* and *Diplosoma*), have been reported to form symbioses with cyanobacteria, although many species in each genus are non-symbiotic (Hirose and Hirose 2007). The symbiosis is thought to have arisen independently in the Didemnidae at least once in each genus (Yokobori et al. 2006; Münchhoff et al. 2007). All symbiotic species are colonial forms from sub-tropical or tropical marine waters and in most cases they harbour unicellular cyanobacteria of the genus *Prochloron* (Fig. 23.17), which contain chlorophyll *a* and *b* but lack phycobilin pigments (Griffiths 2006). However, the unicellular cyanobacterium *Synechocystis trididemni*, a close relative of *Prochloron*, is found in *Trididemnum* species (Münchhoff et al. 2007), and *Trididemnum clinides* harbours three different cyanobionts, two unicellular and one filamentous, none of which seem to be *Prochloron* (Hirose et al. 2009b).

The chlorophyll *d*-containing cyanobacterium *Acaryochloris marina* occurs as an epibiont on the undersides of some didemnid ascidians found on the Great Barrier Reef (Fig. 23.17; Kühl et al. 2005; Larkum and Kühl 2005). The ascidian tissue strongly attenuates visible light, but the far-red light absorbed by chlorophyll *d* penetrates easily and so the underside of the animal is an ideal niche for *Acaryochloris*. However, this cyanobacterium may not be restricted to the surface of tunicates as a recent study found

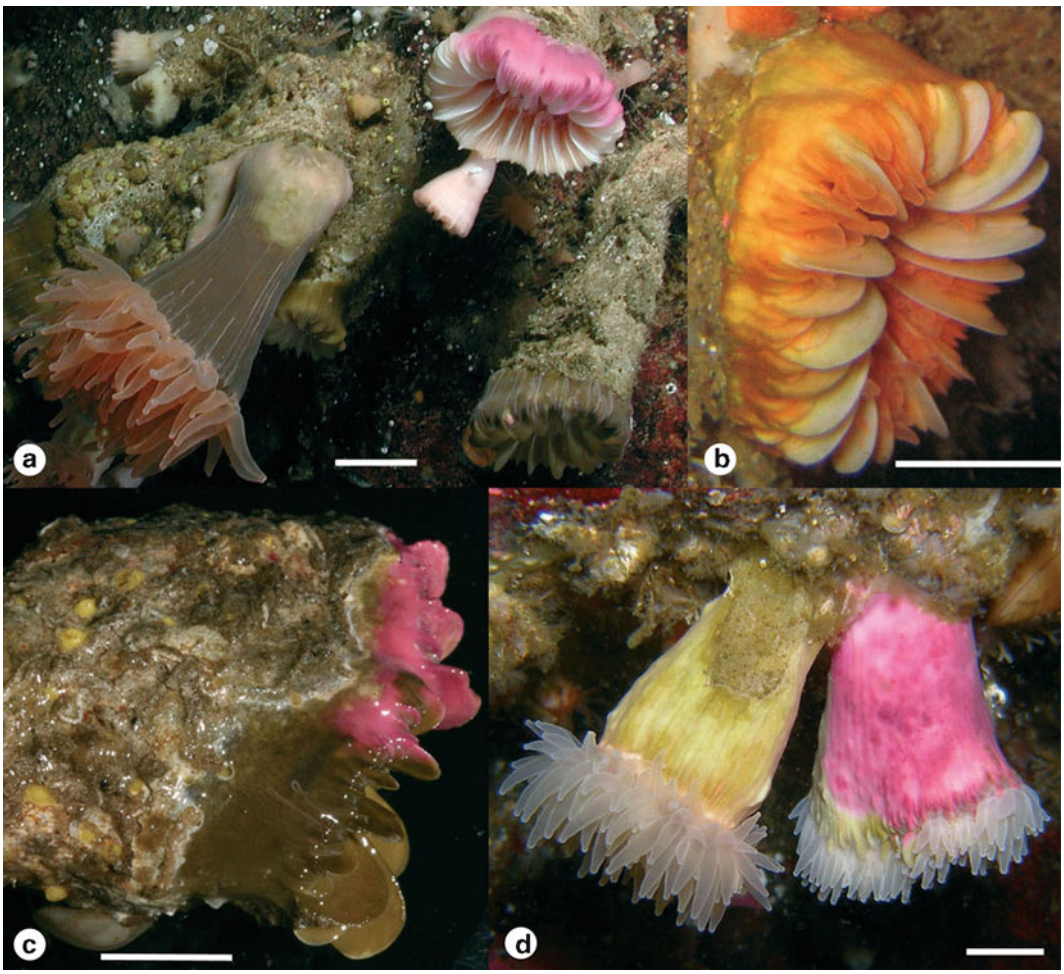

Fig. 23.16 Endolithic cyanobacterium *Plectonema terebrans* in the skeleton of the scleractinian coral *Desmophyllum dianthus* from a Chilean fjord. This cold water coral lacks endosymbiotic zooxanthellae but carries endolithic cyanobacteria and algae in its corallite skeleton. (**a**) *D. dianthus* containing the brownish, filamentous alga *Ostreobium queckettii* (the two lower specimens) or the pinkish filamentous cyanobacterium *Plectonema terebrans* (upper specimen). (**b**) *D. dianthus* appearing yellowish-orange due to the presence of a low density of *O. queckettii*. (**c**) Corallite of *D. dianthus* with pink *P. terebrans* in the upper half and the brownish *O. queckettii* in the lower half. (**d**) *D. dianthus* stained greenish by a medium density of *O. queckettii* (left) and stained pink by *P. terebrans* (right). Bars 10 mm. (Reproduced with permission from Försterra and Häussermann 2008)

Fig. 23.17 Cross-section of the ascidian *Trididemnum paracyclops* showing the green, Chl *a*- and *b*-containing endosymbiotic cyanobacterium *Prochloron didemni*, within internal cavities, and the episymbiotic *Acaryochloris marina*-like cyanobacterium (arrow) on the underside of the animal. The *Acaryochloris* contains Chl *d* which absorbs maximally in the near-infrared region, which is what remains after sunlight has passed through the animal. Bar 5 mm. (Reproduced with permission from Larkum and Kühl 2005)

two *Acaryochloris*-like symbionts in the tunic of *Lissoclinum fragile* (Lopez-Legentil et al. 2011). Epiphytic *Acaryochloris* spp. are also found on the marine red macroalga *Ahnfeltiopsis flabelliformis* (Murakami et al. 2004) and on green and brown marine macroalgae (Ohkubo et al. 2006).

Mutualistic associations between aquatic invertebrates and cyanobacteria or unicellular algae are varied and widespread, particularly in marine subtidal zones of the tropics, such as coral reefs, but are much less common in temperate marine and freshwater environments (Hirose et al. 2009b). These are often low-nutrient waters in which the photoautotrophic symbionts provide the host with a competitive advantage and the associations may make significant contributions

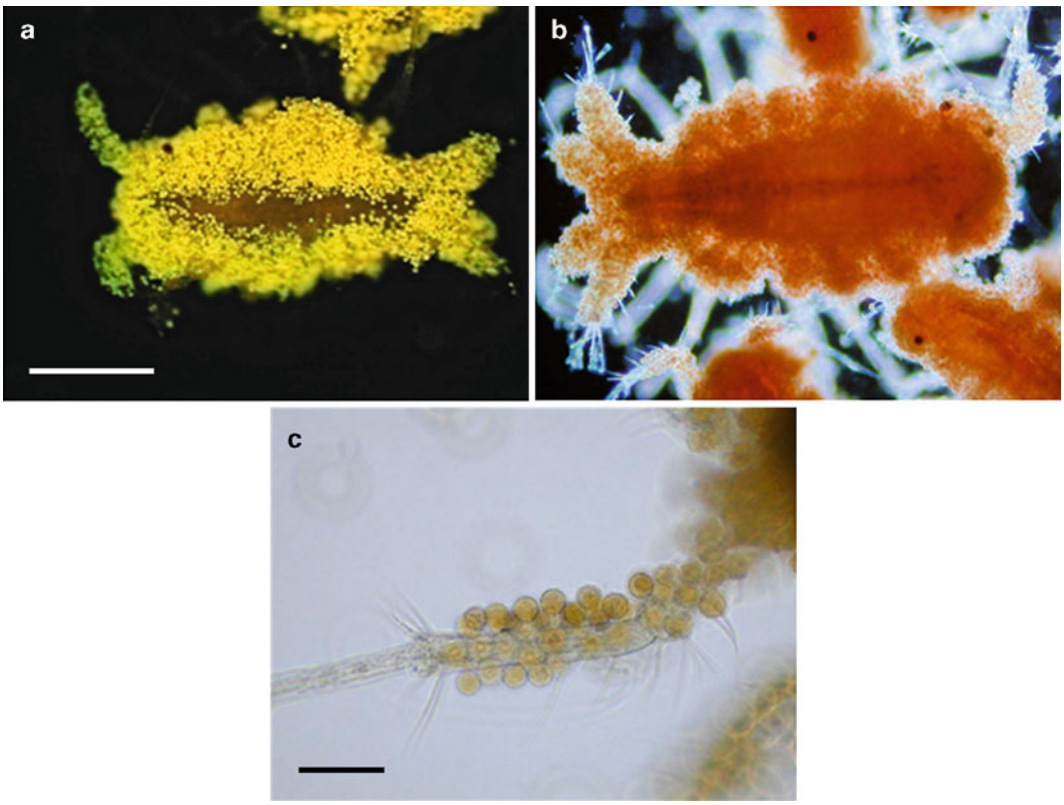

Fig. 23.18 The isopod crustacean *Santia* with episymbiotic unicellular cyanobacteria. (**a**) Epifluorescence photograph of *Santia* with episymbiotic cyanobacteria, individual cells of which can be seen on an isopod antenna in the bright field micrograph in (**c**). (**b**) Dark field image showing the reddish pigmentation of the cyanobacteria. Bars 1 mm in (**a**) and 25 μm in (**c**). (Reproduced with permission from Lindquist et al. 2005)

to the local carbon and N economy (Yellowlees et al. 2008; Venn et al. 2008). Little is known about the specific contribution of cyanobacteria-ascidian symbioses in these environments, although they may be significant. Like many marine invertebrate-bacteria associations, ascidians hosting *Prochloron* are known to produce a range of bioactive compounds, mostly cytotoxic modified peptides such as the patellamides and lissoclinamides, and the source of these is the cyanobiont (Schmidt et al. 2005; Donia et al. 2006; Piel 2006a, b; Jones et al. 2009; Donia et al. 2011a, b; Lane and Moore 2011). These bioactive compounds may act as a chemical defence, which is needed because of the sessile lifestyle of the ascidians.

23.2.5.4 Echiuroid Worms, Isopods, Hydroids and Midge Larvae

Echiuroid worms (spoon worms) are common in the intertidal zone of oceans throughout the world, where they burrow in sand or mud. Two of these soft-bodied, unsegmented worms, *Ikedosoma gogoshimense* and *Bonellia fuliginosa*, have been reported to carry cyanobacteria in their subepidermal connective tissue (Carpenter and Foster 2002; Carpenter 2002). Episymbiotic cyanobacteria are found on another animal host, marine isopods of the genus *Santia* (Fig. 23.18), found around Papua New Guinea (Lindquist et al. 2005). These are non-swimming, slow-moving crustaceans up to 5 mm in length, commonly found in groups of thousands of individuals at depths of 4–45 m. The surface of the isopods is covered by a dense carpet of 20–30 μm diameter unicellular cyanobacteria (Fig. 23.18), together with small (less than 2 μm diameter) morphologically diverse cells. There are red and brown isopods, the former being unpalatable to reef fish and the latter being palatable. The pigmentation is derived from the cyanobacteria (Fig. 23.18b), which, when examined by transmission electron microscopy, show morphological differences between palatable and unpalatable hosts (Lindquist et al. 2005). The cyanobacteria are thought to produce secondary metabolites that render the red form unpalatable and enable it to inhabit exposed, sunlit surfaces to provide the sunlight for their symbionts, which are subsequently consumed by the host. Filamentous cyanobacteria, including *Oscillatoria lutea* and *Spirulina subsalsa*, are found as epibionts on another animal host, the marine hydroid *Eudendrium racemosum* which is widely distributed in the Mediterranean Sea (Romagnoli et al. 2007). The cyanobacteria peak in abundance during the summer months.

An unusual and apparently mutualistic association is found between the cyanobacterium *Nostoc parmeloides* and larvae of the chironomid midge *Cricotopus nostocicola* (see: Adams 2000). The cyanobacterium grows as colonies attached to rocks in mountain streams of North America; the midge larvae live inside the colonies and eat the *Nostoc*, thereby gaining both a food source and protection from predation. Silk from the larvae may help to attach the *Nostoc* colonies to the rocks, but the cyanobacterium appears to benefit in other ways because the rate of photosynthesis is greater in larvae-occupied colonies than in unoccupied ones. This may be a result of the occupied colonies being able to grow further into the water flow, resulting in improved gas exchange, which in turn improves the availability of growth-limiting CO_2 and facilitates the removal of photosynthetically-generated O_2 which might otherwise inhibit N_2 fixation. The larvae appear to stimulate expansion of the *Nostoc* colonies by triggering the formation of motile hormogonia (Sect. 23.4.1.2) which can then migrate and colonise any unoccupied rock surfaces.

23.3 The Symbionts

23.3.1 Cyanobacteria

The range of cyanobacterial symbionts encompasses all morphological forms, from unicellular to filamentous non-heterocystous and filamentous heterocystous, although certain forms are favoured by particular hosts (Rasmussen and Nilsson 2002; Rasmussen and Johansson 2002). For example, most plants favour heterocystous cyanobionts of the genus *Nostoc* because members of this genus can produce the specialised, motile filaments known as hormogonia which are the infective agents in these symbioses (Meeks et al. 2002; Bergman et al. 2008a, b; Sect. 23.4.1.2). However, the ability to form hormogonia *per se* is not sufficient for symbiotic competence as hormogonia-forming cyanobacteria of genera such as *Fischerella* have never been found in plant symbioses, although they are found in lichens.

23.3.1.1 Mosses, Hornworts and Liverworts

In the epiphytic moss associations the feather moss *Pleorozium schreberi* can be colonised by *Nostoc* (DeLuca et al. 2002, 2007; Houle et al. 2006), *Stigonema* (Houle et al. 2006; DeLuca et al. 2007) and *Calothrix* (Gentili et al. 2005; DeLuca et al. 2007). When grown free-living, away from the moss, the *Nostoc* and *Calothrix* have different temperature optima for N_2 fixation, which may help explain seasonal heterogeneity for N_2 fixation in the cyanobacteria-*P. schreberi* association (Gentili et al. 2005). A N_2 fixing association can be reconstituted using both cyanobacterial isolates and non-N_2 fixing moss (Gentili et al. 2005). Two additional mosses, *Hylocomnium splendens* and *Ptillium crista-castrensis*, associate with *Nostoc* and *Stigonema* (Houle et al. 2006), and there have also been reports of cyanobacteria from other genera, including *Phormidium*, *Microcystis* and *Oscillatoria*, as epiphytes on mosses in Antarctica (Solheim et al. 2004).

The major cyanobionts of the liverworts and hornworts are *Nostoc* (Rasmussen and Nilsson 2002; Adams 2002a, b; Adams et al. 2006; Bergman et al. 2007a, b; Adams and Duggan 2008), although other hormogonia-forming cyanobacterial genera, such as *Calothrix* and *Chlorogloeopsis*, can be induced to infect liverworts in the laboratory and a single strain of *Calothrix* has been isolated from a field sample of the hornwort *Phaeoceros* (see: Adams 2002a, b). A variety of molecular techniques, including comparison of $tRNA^{Leu}$ (UAA) intron sequences (Costa et al. 2001), PCR amplification of cyanobiont DNA flanking the 16S–23S rRNA internal transcribed spacer regions, and pyrolysis mass spectrometry of isolated cyanobionts (Adams 2002a, b; Adams and Duggan 2008), have demonstrated that a wide variety of *Nostoc* strains can infect a single thallus in the field. Costa et al. (2001) found the same *Nostoc* strain shared by thalli growing 2,000 m apart, whereas others have failed to find the same strain in thalli at different sites (Adams 2002a, b; Adams and Duggan 2008). Nested PCR of the $tRNA^{Leu}$ (UAA) intron was used by Rikkinen and Virtanen (2008) to demonstrate that the primary symbiont of both *Blasia pusilla* (from Finland) and *Cavicularua densa* (from Japan) were closely related *Nostoc* strains which belonged to a specific group of symbiotic *Nostoc* strains. They concluded that, although more than one *Nostoc* genotype is often found within a single liverwort thallus, some symbiotic strains are dominant and widespread.

23.3.1.2 Cycads

Cycad cyanobionts are usually *Nostoc* although *Calothrix* have been reported on several occasions (Costa and Lindblad 2002; Rasmussen and Nilsson 2002; Gehringer et al. 2010; Thajuddin et al. 2010). Using the $tRNA^{Leu}$ (UAA) intron sequence as a genetic marker (Costa et al. 2002), Costa et al. (2004) found a single cyanobacterial strain was often present in a single coralloid root, or even in a single plant. However, a study using PCR fingerprinting with primers derived from short tandemly repeated repetitive (STRR) sequences reported multiple strains in single plants or even in single roots (Zheng et al. 2002). These varying results may reflect differences in the molecular methods used to characterise cyanobionts and it may be that the use of the $tRNA^{Leu}$ (UAA) intron sequence is more reliable than PCR fingerprinting (Costa et al. 2004). An analysis of 16S rRNA sequences and STRR PCR fingerprints of cyanobionts from three *Cycas* species from four botanical gardens in India found a diversity of *Nostoc* spp. (Thajuddin et al. 2010). A similar analysis of the 16S rRNA gene sequences of

cyanobacteria isolated from *Macrozamia* spp. throughout Australia found that the predominant *Nostoc* sp. was present in 18 root samples from 14 different *Macrozamia* spp. from a broad range of environments (Gehringer et al. 2010). The authors concluded that there was negligible host specialisation by cyanobionts in *Macrozamia* in the field.

23.3.1.3 *Gunnera*

Gunnera exerts a high level of selectivity over its symbiotic partners, only *Nostoc* spp. being found as cyanobionts, and only those strains capable of high levels of differentiation into hormogonia (Sect. 23.4.1.2; Bergman et al. 2007a, 2008a). However, this characteristic alone is not enough to guarantee a strain's symbiotic competence, as some strains forming high levels of motile hormogonia are still incapable of generating stable symbioses with *Gunnera* (Nilsson et al. 2006). While selection for *Nostoc* is highly specific, there is a growing body of work showing that *Nostoc* strains found in symbiotic relationships with *Gunnera* show high levels of genetic variability. Different *Nostoc* strains are found between different *Gunnera* plants in a similar area, and different strains of *Nostoc* are found in different species of *Gunnera* (Nilsson et al. 2000; Guevara et al. 2002; Rasmussen and Svenning 2001). Furthermore, when phylogenetic analysis is applied, it is clear that *Gunnera* cyanobionts are not all members of the same species (Svenning et al. 2005).

Closely-related *Nostoc* strains show differing abilities to infect *Gunnera* (Papaefthimiou et al. 2008a), implying that chemical signalling between the plant and bacterial cells is an essential component of the initiation of a successful symbiosis. Symbiotically-competent strains of *Nostoc* are also attracted to the crushed extracts of a variety of plants, including some which do not form symbioses (Nilsson et al. 2006). However, only symbiotically-competent species are found deep in the channels of the *Gunnera* gland structure, suggesting there is a highly specific selective mechanism within the gland itself.

23.3.1.4 *Azolla*

The *Azolla* cyanobiont was first described by Strasburger as *Nostoc* "strings" in 1873 and later re-named *Anabaena azolla*e Strasburger in 1884 (see: Pabby et al. 2004b). Although widely acknowledged as belonging to the Nostocales, there has been much debate over the correct generic assignment of the cyanobiont (Bergman et al. 2007a, b). This confusion has arisen in part because the leaf cavities are occupied by major (primary) cyanobionts (apparently unable to grow outside the symbiosis), along with minor (secondary) culturable cyanobionts (Papaefthimiou et al. 2008a, b; Sood et al. 2008a, b). The culturable cyanobionts are phenotypically and molecularly similar to each other but significantly different from the major cyanobiont.

Azolla is unique among plant-cyanobacterial associations in that the cyanobacterium never leaves its host, negating the need for re-infection of each new generation. Attempts to induce free-living strains of *Anabaena azollae* to re-infect *Azolla* cured of its cyanobiont have been largely unsuccessful and it seems impossible to cultivate the major cyanobiont separately (Lechno-Yossef and Nierzwicki-Bauer 2002; Pabby et al. 2004a), implying that one or more of the specific qualities required for free-living growth has been lost during co-evolution with the host (Rasmussen and Nilsson 2002; Sood et al. 2008b; Ran et al. 2010). It is not surprising then, that there appears to be little or no genetic diversity of the cyanobiont within a particular species of *Azolla*, indicating high host specificity, with each *Azolla* species harbouring a specific cyanobacterial strain irrespective of geographical origin.

Although the major *Azolla* symbiont has traditionally been assigned to the genus *Anabaena*, this has often been questioned, some suggesting that the cyanobiont is neither *Anabaena* nor *Nostoc* (Baker et al. 2003). Nevertheless, analyses of 16S rRNA gene sequences amplified by PCR from cyanobionts freshly recovered from *Azolla* suggest that these cyanobionts are most phylogenetically related to strains of the genus *Anabaena* (Svenning et al. 2005; Papaefthimiou et al. 2008a, b). However, in the draft genome sequence of the *Azolla* cyanobiont which has recently become available (http://genome.jgi-psf.org/anaaz/anaaz.home.html; Ran et al. 2010) the cyanobiont is referred to as *Nostoc azollae* 0708, although the closest phylogenetic relatives appear to be *Raphidiopsis brookii* D9 and *Cylindrospermopsis raciborskii* CS-505, the two filamentous cyanobacteria with the smallest known genomes (Ran et al. 2010). By contrast, the *Azolla* cyanobiont shares the highest number of protein groups with *Nostoc* PCC 7120, *Anabaena variabilis* ATCC 29413 and *Nostoc punctiforme* PCC 73102, with the last of these having the highest number of protein groups shared exclusively with *Nostoc azollae* (Ran et al. 2010). The primary cyanobiont has a small (5.49 Mb) genome, comprising one chromosome and two plasmids, and contains 5,357 coding sequences of which 3,668 have intact open reading frames while the rest are pseudogenes (Ran et al. 2010). High numbers of pseudogenes are a trait associated with endosymbionts in sheltered environments where the likelihood of encountering foreign DNA is low. The large proportion of pseudogenes (31.2%) found within all genomic functions present in the genome of the cyanobiont suggests a high level of gene erosion (Ran et al. 2010).

The taxonomic status of the minor cyanobiont population amongst *Azolla* species has received relatively little attention. The most recent analysis suggests the cyanobiont belongs to a distinct group, genetically distinct from either free-living *Nostoc* or *Anabaena* (Sood et al. 2008a) and supports previous genetic, morphological and biochemical observations

that the cyanobacteria (even the minor cyanobionts) associating with *Azolla* are peculiar to the fern (Pabby et al. 2003; Sood et al. 2008a). The continued presence of the minor symbiont in *Azolla* implies an important role in the symbiosis, but the nature of this remains a mystery.

23.3.1.5 Lichens

The genus *Nostoc* also provides many of the cyanobionts of lichens, although many other genera of both filamentous and unicellular cyanobacteria are also represented (see Rikkinen 2002 for a comprehensive list). However, until the application of molecular techniques the identification of lichen cyanobionts had relied on morphological characteristics which, even for the heterocystous cyanobacteria, are not always reliable (see for example Lücking et al. 2009), especially as morphologies can differ significantly in the free-living and symbiotic states (Sect. 23.6.2). Many cyanobionts are members of heterocystous genera including *Nostoc*, *Scytonema*, *Calothrix*, *Dichothrix*, *Stigonema*, *Tolypothrix*, and *Fischerella* (Figs. 23.9 and 23.10; Rikkinen 2002; Schultz and Büdel 2002; Sassaki et al. 2005; Schultz 2007; Muggia et al. 2011; Fedrowitz et al. 2011) and all of these can fix N_2. Unicellular cyanobionts include members of the genera *Gloeocapsa*, *Gloeothece*, *Hyella, Chroococcidiopsis, Myxosarcina* and *Synechocystis* (Fig. 23.10c), although their exact taxonomic status is often uncertain (Rikkinen 2002; Schultz and Büdel 2002; Adams et al. 2006). Some unicellular cyanobionts have been shown to fix N_2 when lichenised, although this occurs during the light, rather than in the dark as with free-living unicellular cyanobacteria (Crittenden et al. 2007).

Although some lichens have evolved means, such as soredia and isidia, for the co-dispersal of fungus and photobiont, most reproduce via sexually-produced ascospores which are not associated with photobiont cells. The germinating ascospores therefore have to locate a suitable photobiont in the environment. This need to find a suitable algal or cyanobacterial partner in the near vicinity of the germinating spore may be expected to limit the scope for specialisation of the partners since this would reduce the chances of finding the right partner (O'Brien et al. 2005; Hill 2009). Molecular phylogenetic techniques have made it possible to compare cyanobacterial strains from a wide range of lichens and look for specificity between fungus and cyanobiont. The use of the nucleotide sequences of the $tRNA^{Leu}$ (UAA) intron and 16S rDNA to compare the *Nostoc* cyanobionts of different lichens has revealed wide genetic variation (Paulsrud et al. 2000, 2001; Paulsrud 2001; Rikkinen 2002; Oksanen et al. 2002). These earlier molecular phylogenetic studies found that cyanobionts could be shared between related hosts such as members of the Nephromataceae (Paulsrud et al. 2000; Summerfield et al. 2002; Rikkinen 2002, 2003; Rikkinen et al. 2002; Lohtander et al. 2003; Wirtz et al. 2003). Later studies showed that symbiont sharing was common even between unrelated hosts and not just in lichens from extreme environments (such as the Antarctic) where the choice of photobiont might be low (Wirtz et al. 2003), but also in those from temperate regions (O'Brien et al. 2005; Summerfield and Eaton-Rye 2006).

The two primary factors which might influence the selection of the cyanobiont by the lichen fungus are habitat ecology and fungal taxonomy, yet distinguishing between these using molecular phylogenetic techniques has proved difficult and controversial. Earlier studies concluded that it was primarily the taxonomic identity of the lichen, rather than its geographic origin, that determined its choice of cyanobiont (Paulsrud et al. 2000; Summerfield et al. 2002). However, subsequent studies concluded that geographic origin was the more important factor. For example, unrelated cyanolichens were shown to share the same cyanobiont and these lichens often formed characteristic communities or "guilds" that shared a common habitat, so epiphytic cyanolichens associated with old-growth forests formed the *Nephroma* guild and many predominantly terrestrial cyanolichens formed the *Peltigera* guild (Rikkinen 2002, 2003; Rikkinen et al. 2002). The cyanobionts within these guilds are closely related and form two clades, referred to by Rikkinen et al. (2002) as *Nostoc* groups A and B, which are cyanobionts of, respectively, the *Nephroma* and the *Peltigera* guilds.

This concept of "pools" of symbiotically-competent cyanobacteria being available for lichenisation by mycobionts in the vicinity gained further support from the work of Summerfield and Eaton-Rye (2006), who concluded that there was no correlation between mycobiont diversity and cyanobiont choice in the *Pseudocyphellaria* species examined. Following more comprehensive surveys using sequences of $tRNA^{Leu}$, 16S rDNA, *rbcL* and *rbcX* as genetic markers, Stenroos et al. (2006) and Myllys et al. (2007) concluded that, although many lichen fungi were indeed selective towards their *Nostoc* cyanobionts, this correlated primarily with the fungal taxa, rather than the terrestrial or epiphytic nature of the lichens. However, a different conclusion was reached by Elvebakk et al. (2008) who examined phylogenetic patterns of the *Nostoc* symbionts of both bi- and tri-partite lichens of *Pannaria* from seven countries in northern and southern hemispheres. They broadly supported the idea that selectivity of *Nostoc* strains resulted primarily (although not exclusively) from ecological influences, but there was a transition between the *Nephroma* and *Peltigera* guilds (Elvebakk et al. 2008). Further support for lichen guilds was presented by Lücking et al. (2009) based on their phylogenetic analysis of what were thought to be *Scytonema* cyanobionts in a range of tropical lichens. The cyanobionts, which were shared by at least four genera of lichen mycobionts, formed a distinct clade which was more closely related

to *Nostoc*, *Anabaena*, *Fischerella* and *Hapalosiphon* than to *Scytonema*, and which they named *Rhizonema*.

In summary, although there is still some dispute about whether it is ecology or genetics which most influences the mycobiont's choice of cyanobacterial partner, it is clear that mycobionts are selective in their choice of cyanobionts, whereas many cyanobionts associate with a range of often unrelated mycobionts.

23.3.1.6 *Geosiphon pyriformis*

In *Geosiphon pyriformis* only *Nostoc* strains, usually referred to as *Nostoc punctiforme*, can be cyanobionts (Kluge et al. 2002; Adams et al. 2006). In its natural environment *Geosiphon* is found together with the liverwort *Blasia*, the hornwort *Anthoceros*, and the moss *Dicranella* (Kluge et al. 2002). Both *Blasia* and *Anthoceros* have their own *Nostoc punctiforme* symbionts and these can be recognised and incorporated by *Geosiphon* (Adams et al. 2006). There are also endosymbiotic bacteria (referred to as bacteria-like organisms, BLOs) within the fungus and, unlike the cyanobiont, these are not surrounded by a host membrane (Adams et al. 2006).

23.3.1.7 Diatoms

Comparison of *nifH*, *hetR* and 16S rRNA sequences of the *Richelia intracellularis* cyanobionts of the diatoms *Hemiaulus hauckii* from the North Atlantic and *Rhizosolenia clevii* from the North Pacific has shown that they are different species (Foster and Zehr 2006). This work also confirmed that the cyanobiont of *Chaetoceros*, previously referred to as both *Richelia intracellularis* and *Calothrix rhizosoleniae*, is closely related to, but distinct from, the *Richelia* symbionts of *Hemiaulus hauckii* and *Rhizosolenia clevii*. Even within the Mediterranean Sea two phylogenetically-discrete populations of *R. intracellularis* have been found in *Hemiaulus hauckii*, seemingly kept apart by local water circulation patterns (Bar Zeev et al. 2008).

Phylogenetic analysis of the 16S rDNA sequence of the coccoid cyanobiont of the diatom *Climacodium frauenfeldianum* from the Atlantic and Pacific Oceans has shown that it is closely related to the N_2 fixing *Cyanothece* ATCC 51142 (Carpenter and Janson 2000; Janson 2002) and to free-living unicellular N_2 fixing cyanobacteria found in the tropical North Atlantic Ocean (Falcón et al. 2002). Similarly, genome sequence analysis of the cyanobiont (the so-called spheroid bodies) of the marine pennate diatom *Rhopalodia gibba* has confirmed that it also has a close relationship to *Cyanothece* ATCC 51142 (Prechtl et al. 2004; Kneip et al. 2008). However, the genome of this cyanobiont has undergone significant changes, including the elimination, fusion and truncation of genes, and the accumulation of deleterious mutations in genes for cell wall biosynthesis, confirming the speculation that it is an obligate endosymbiont and that it must be transmitted vertically (Kneip et al. 2008).

23.3.1.8 Dinoflagellates, Radiolarians and Tintinnids

A range of unicellular cyanobacterial symbionts has been reported in members of the dinoflagellate genera *Ornithocercus*, *Histioneis*, *Parahistioneis*, *Citharistes*, *Dinophysis* and *Amphisolenia* (Fig. 23.4; Carpenter 2002; Foster et al. 2006a, b; Tarangkoon et al. 2010). RT-PCR analysis of 16S rRNA sequences of cyanobionts from *Citharistes*, *Ornithocercus* and *Histioneis* has shown them to be related to *Prochlorococcus*, although additional 16S rRNA sequences from *Citharistes* spp. were related to *Synechococcus* (Foster et al. 2006b). The cyanobionts of the two radiolarians, *Spongostaurus* and *Dictyocoryne truncatum*, are thought to be related to *Prochlorococcus* (Foster et al. 2006a, b). The open-ocean tintinnid *Codonella* hosts unicellular cyanobacterial symbionts closely related to *Synechococcus* (Carpenter and Foster 2002; Foster et al. 2006b). It seems that many diverse open-ocean hosts, encompassing dinoflagellates, tintinnids and radiolarians, house cyanobionts related to the unicellular, free-living marine *Synechococcus* and *Prochlorococcus*, indicating a relatively low degree of specificity in these symbioses (Foster et al. 2006b).

23.3.1.9 Sponges

Cyanosponges contain both unicellular and filamentous cyanobacteria as symbionts (Hentschel et al. 2006; Usher 2008). Unicellular cyanobacteria include *Aphanocapsa feldmannii*, *A. raspaigellae*, *Synechococcus* spp., *Prochloron* spp. and *Synechocystis trididemini*, whereas filamentous cyanobacteria include *Oscillatoria spongeliae*, which occurs as closely-related strains in a wide range of sponges (Thacker and Starnes 2003; Hentschel et al. 2006; Hill et al. 2006; Usher et al. 2006; Thacker et al. 2007; Zhu et al. 2008; Usher 2008; Erwin et al. 2012). 16S rRNA studies have revealed that *Aphanocapsa feldmannii* symbionts are in fact *Synechococcus* (Hentschel et al. 2002; Gómez et al. 2004; Usher et al. 2004a). Analysis of high molecular weight DNA from the sponge *Halichondria* has identified sequences showing high homology with cyanobacteria from the unicellular genus *Synechococcus* and the filamentous, heterocystous genus *Nostoc* (Ouyang et al. 2010).

However, the most prevalent sponge cyanobiont is probably *Synechococcus spongiarum* which has been isolated from taxonomically-diverse hosts from wide-ranging geographical locations and appears to be specifically adapted to the host sponges (Usher et al. 2004b; Steindler et al. 2005; Thacker 2005; Oren et al. 2005; Erwin and Thacker 2007, 2008a, b; Hardoim et al. 2009). Comparison of cyanobiont 16S rDNA sequences has provided invaluable information on cyanobiont identity and diversity. In general, specific sponges

host specific cyanobionts, both across wide geographical locations and when sponges hosting alternative cyanobionts are close by (Hentschel et al. 2002; Usher 2008). For example, different sponges have been shown to always host the same distinct strain of *Oscillatoria spongeliae* (Thacker and Starnes 2003; Ridley et al. 2005a) and the cyanobionts of *Chondrilla australiensis* from tropical and temperate regions are from the same clade (Usher et al. 2004a). Individual sponges occasionally host two or more cyanobionts (Usher et al. 2004a; Ridley et al. 2005b; Steindler et al. 2005).

Relatively few sponges have been studied with respect to the mode of transmission of cyanobionts, which could occur either vertically, directly from parent to offspring, or horizontally, by the offspring obtaining the cyanobiont from the environment (Taylor et al. 2007; Usher 2008). Vertical transmission has been shown in the oviparous sponge *Chondrilla australiensis* (Usher et al. 2001, 2005; Usher 2008) and the viviparous *Diacarnus erythraeanus* (Oren et al. 2005). Occasional horizontal transfer may also occur, possibly facilitated by the expulsion of cyanobionts along with eggs during spawning (Taylor et al. 2007; Usher 2008). Remarkably, cyanobacteria have been found in both eggs (Usher et al. 2004b) and sperm (Usher et al. 2005) of *Chondrilla australiensis*. However, even in this case occasional females do not vertically transmit their cyanobionts at all and offspring may acquire new cyanobionts from the water (Usher et al. 2005). Symbiotic cyanobacteria have also been seen in the oocytes of the marine sponge *Chondrilla nucula* (Maldonado 2007) and in buds produced by the marine sponge *Tethya orphei* (Gaino et al. 2006).

23.3.1.10 Corals

The coccoid cyanobionts of the coral *Montastreae cavernosa* are most closely related to either *Synechococcus* or *Prochlorococcus* in the order Chroococcales (Lesser et al. 2004, 2007). Among the partial *nifH* sequences obtained by PCR amplification of DNA from tissues of *Montipora capita* and *M. flabellata* were some most closely resembling those of the cyanobacterial genus *Myxosarcina* (Olson et al. 2009). In addition, unidentified cyanobacterial DNA sequences have been detected in the metagenome of the bacterial community from the coral *Porites compressa* (Thurber et al. 2009). Terminal restriction fragment length polymorphism analysis of the microbial population of the coral *Montastraea annularis* identified an abundant cyanobacterium (cyanobacterium CD1C11) normally associated with coral black band disease (BBD), but in this case found in many samples of healthy coral (Klaus et al. 2007). BBD affects corals worldwide and is characterised by a microbial mat dominated by cyanobacteria that were initially identified as *Phormidium corallyticum*. However, later analysis of BBD mats from three different locations revealed them to contain cyanobacteria from at least three different taxa of the order Oscillatoriales (Frias-Lopez et al. 2003; Myers and Richardson 2009). It isn't clear if cyanobacterium CD1C11 is epibiotic or endobiotic on healthy *Montastraea annularis*.

23.3.1.11 Ascidians

The most frequent cyanobionts in the colonial ascidians of the family Didemnidae are unicellular *Prochloron* spp., which contain chlorophyll *a* and *b* but lack phycobilin pigments (Griffiths 2006; Hirose and Hirose 2007; Hirose et al. 2009b; Lopez-Legentil et al. 2011). However, the unicellular, coccoid cyanobacterium *Synechocystis trididemni*, shown by molecular phylogenetic analysis to be a close relative of *Prochloron* (Shimada et al. 2003), has been reported in *Trididemnum* species (Münchhoff et al. 2007; Lopez-Legentil et al. 2011). *Synechocystis trididemni*, or a closely-related species, is also the dominant cyanobiont in *Trididemnum clinides* from Japan, but this ascidian harbours two additional cyanobionts, a possibly new non-*Prochloron* species and a filamentous, non-heterocystous cyanobacterium, possibly an *Oscillatoria* sp. (Hirose et al. 2009b).

Phylogenetic analysis of *Prochloron* spp. from a wide range of didemnid ascidians in widespread geographical locations has revealed little genetic variation and no relationship between phylogeny and geographic location, implying a low level of host specificity (Münchhoff et al. 2007). The *Prochloron*-didemnid symbiosis is generally considered to be obligate for both partners because *Prochloron* has never been grown in culture (apart from one unconfirmed early report) and has only very rarely been seen free-floating away from the host. Indeed, in many photosymbiotic didemnids the cyanobiont is passed vertically from mother to larva, although the exact mechanism of transmission varies in different didemnids (Hirose 2000; Hirose et al. 2005, 2006c; Hirose and Hirose 2007; Kojima and Hirose 2010). However, *Prochloron* 16S rRNA gene sequences have been found in living stromatolites in an environment free of ascidians, implying that *Prochloron* can exist free of its host (Burns et al. 2004). Similarly, recent sequencing of the genome of the *Prochloron didemni* symbiont from *Lissoclinum patella* revealed a complete set of metabolic genes, implying that the cyanobacterium can reproduce outside the host (Donia et al. 2011).

Colonial ascidians from the Mediterranean Sea have recently been found with a complex epibiotic population of cyanobacteria from genera *Planktothricoides, Synechococcus, Phormidium* and *Myxosarcina* (Martinez-Garcia et al. 2011). Cyanobacteria resembling the chlorophyll *d*-containing *Acaryochloris marina* are found as epibionts on the undersides of the didemnid ascidians *Lissoclinum patella*, *Trididemnum paracyclops* and *Diplosoma similis*, but are not found within the ascidian tissue (Kühl et al. 2005; Larkum and Kühl 2005). However, two *Acaryochloris marina*-like cyanobacteria have recently been found in the tunic of both adults and larvae of *Lissoclinum fragile* from the Bahamas (Lopez-Legentil et al. 2011).

23.3.1.12 Isopods

The surface of small, marine crustacean isopods of the genus *Santia* is covered by a dense carpet of 20–30 μm diameter unicellular cyanobacteria, together with small (less than 2 μm diameter) morphologically-diverse cells (Fig. 23.18; Lindquist et al. 2005). Comparison of PCR-amplified DNA-dependent RNA polymerase complex (rpoC1) clones from symbionts has revealed strong similarity with *Synechococcus*, *Synechocystis* and *Prochlorothrix* (Lindquist et al. 2005). The authors concluded that the *Synechococcus* were the smaller cells found alongside the large symbiont and were environmentally derived, whereas the large-celled symbionts were *Synechocystis* and were probably vertically transmitted.

23.3.2 Bacteria

Apart from *Azolla*, which is described below, little is known of the possible involvement of bacteria in cyanobacterial symbioses. In *Gunnera*, microorganisms other than cyanobacteria are excluded from the symbiotic structures occupied by the cyanobionts, whereas in sponges for example, complex populations of bacteria are also present.

The leaf cavities in all *Azolla* species house a substantial range of endosymbiotic bacteria, often referred to as bactobionts or eubionts. Bacteria are also found at the shoot apex, in the indusium chambers of both mega and microsporocarps and germlings, and even inside *Azolla* root and stem tissues (Bergman et al. 2007a; Zheng et al. 2009a, b). Older leaves in general harbour larger bacterial communities, although the number and type of bacteria present varies depending on the *Azolla* species. In some cases the bacterial population has been found to be equal to the number of cyanobacterial cells present. Estimates suggest that only 1% of the bacteria are cultivable, implying that the isolates identified to date represent only part of the bacterial population able to enter the *Azolla*-cyanobacterium associations (Zheng et al. 2009a, b). The significance of these bacteria, which have also been found in cyanobiont-free *Azolla* spp. (reviewed by Zheng et al. 2009a), is unclear, although their presence throughout the life cycle of *Azolla* would imply an important contribution to the association. There is evidence that some may fix N_2 and also contribute to the polysaccharide-rich mucilaginous matrix associated with *Azolla* leaf cavities and possibly also to that found in the indusium chambers of the sporocarps and in the sporeling (Zheng et al. 2009a, b). Early work has also shown that cultured *Arthrobacter* isolated from *Azolla* secrete auxin when supplied with the precursor tryptophan, raising the possibility that the bacterial endosymbionts release the plant hormone in the symbiosis with *Azolla* (Lechno-Yossef and Nierzwicki-Bauer 2002).

23.4 Host-Cyanobacteria Interactions Prior to Infection

23.4.1 What Makes a Successful Cyanobiont?

In the plant symbioses successful cyanobionts possess two significant characteristics – they are capable of forming motile hormogonia, which are essential for successful plant invasion, and heterocysts, which are required for N_2 fixation and the establishment of a functional symbiosis. Hormogonia are particularly important in the plant associations because the immotility of the host means that the cyanobacteria must find the plant. By contrast, motile hosts such as diatoms and sessile filter-feeders such as sponges can locate and capture potential symbionts from the surrounding water. Indeed, for many of the non-plant cyanobacterial symbioses, hormogonia are not required and the cyanobionts are often incapable of forming hormogonia or heterocysts; their role is then usually the provision of fixed carbon for the association.

For hormogonia to successfully locate and invade the plant structures that will house the symbiotic colonies they must possess at least two characteristics in addition to motility. Firstly, they must be able to adhere to, and perhaps specifically recognise, the host plant surface; external filamentous protein structures known as pili are essential for this. Secondly, they must be able to sense chemoattractants released by the host. Finally, in addition to the ability to form heterocysts and hormogonia plant cyanobionts need to be facultative heterotrophs. In many of the plant symbioses the cyanobiont is found in locations (e.g. cycad roots and *Gunnera* stem glands) that receive little, if any, light and so it must be able to grow heterotrophically on fixed carbon supplied by the plant partner.

23.4.1.1 Heterocysts

Many cyanobionts, especially those of plants, are heterocystous cyanobacteria from the genus *Nostoc* and their primary role is to provide fixed nitrogen for the symbiotic partnership, although in some cases, such as two-membered cyanolichens, they also provide fixed carbon. Heterocysts are the sites of N_2 fixation, providing a suitable microoxic environment for nitrogenase to function. They develop in response to N limitation and their frequency in symbiosis is often greatly elevated above that in free-living cyanobacteria (Sect. 23.6.2.2) resulting in an increased rate of N_2 fixation. However, heterocysts are important for another reason – they are produced by all cyanobacteria of the families Nostocaceae and Stigonemataceae, and it is these cyanobacteria that are capable of the production of a particular type of motile hormogonia, the formation of which is characterised by rapid cell division resulting in a decrease in cell size (Sect. 23.4.1.2). It is these hormogona that are the infective agents in many plant symbioses.

23.4.1.2 Hormogonia

Cyanobionts from genera such as *Nostoc*, which are sessile for most of their life cycles, have the ability to convert immotile vegetative filaments into short, specialised motile filaments known as hormogonia, that serve as both a means of dispersal and as the infective agents in most cyanobacteria-plant symbioses (Meeks 2003, 2009; Meeks and Elhai 2002; Bergman et al. 2007a). There are a variety of environmental factors that trigger hormogonia development (Meeks et al. 2002; Meeks and Elhai 2002; Meeks 2009) and these include chemicals released by plants (Sect. 23.4.2). Once inside the plant the hormogonia lose motility and form the heterocysts that are essential for the establishment of a successful, N_2 fixing symbiosis.

In heterocystous cyanobacteria the first visible event in hormogonia development is a round of very rapid cell divisions, which occurs in all cells of a filament, without cell growth, resulting in a large decrease in cell volume (Meeks and Elhai 2002; Bergman et al. 2008a; Meeks 2009). Subsequently, the vegetative cell-heterocyst junctions become narrowed and break, resulting in the loss of the heterocysts and the release of the short interheterocyst sections of filament, which at the same time acquire motility and are now hormogonia. Because hormogonia lack heterocysts they are only a transient stage in the *Nostoc* life-cycle, soon loosing motility and returning to vegetative growth. At this point they begin to develop heterocysts, initially from the terminal cell at each end of the hormogonium (at which point the hormogonium is sometimes referred to as a primordium), but later at intercalary positions once vegetative cell division increases the length of the filament. The surface of *Nostoc punctiforme* hormogonia is covered with pili (fimbriae) which are clearly important for the infection process and may be involved in motility, host recognition and surface attachment (Duggan et al. 2007).

23.4.1.3 Pili

The cell surface of hormogonia is covered with filamentous, protein structures known as Type IV pili (Tfp) which are found in many bacteria and are involved in a wide range of processes including adhesion, pathogenesis, DNA uptake and motility (Mattick 2002; Nudleman and Kaiser 2004; Burrows 2005). In *N. punctiforme* the non-motile vegetative filaments lack pili, whereas the surface of motile hormogonia is covered with abundant, peritrichously-arranged pili (Duggan et al. 2007). Tfp are involved in the motility of some unicellular cyanobacteria (Bhaya 2004), but it isn't known if they are also involved in the gliding motility of filamentous cyanobacteria, or of hormogonia. Mutation of two *N. punctiforme* genes, homologues of *pilT* and *pilD* which are thought to be involved in Tfp structure and function, has adverse effects on the ability of the mutant hormogonia to infect the liverwort *Blasia* (Duggan et al. 2007). Because of inconsistent motility in the wild-type hormogonia it isn't possible to determine from the data of Duggan et al. (2007) if the reduced hormogonia symbiotic competence in the Tfp mutants is due to loss of motility (and hence chemotaxis; next section), or to loss of some other trait, such as host recognition or adhesion to the plant surface. Further support for the involvement of pili in symbiotic interactions comes from a proteomic and transcriptional analysis of *Nostoc* hormogonia formation which revealed strong upregulation of a homologue of *pilQ* (Klint et al. 2006), upstream of which in the *Nostoc punctiforme* genome are three ORFs homologous to *pilM*, *pilN* and *pilO*, all of which are involved in pilus formation (Meeks et al. 2001).

23.4.1.4 Chemotaxis

The production of motile hormogonia is essential for the efficient infection of many plant hosts, yet some *Nostoc* strains produce motile hormogonia but do not show symbiotic competency, so there must be additional characteristics required to ensure successful infection. One such characteristic is likely to be the ability to sense and respond to plant-derived chemoattractants to enable the hormogonia to locate and invade host symbiotic structures. This sensory capacity is reflected in the gene expression changes that accompany the development of hormogonia, induced by either N starvation or exposure to hormogonia-inducing factors (Sect. 23.4.2) released by plants (Campbell et al. 2007, 2008). Within 24 h of inducing hormogonia formation in *N. punctiforme* the transcription of 944 genes is upregulated and 856 downregulated; this is fivefold greater than the number of transcriptionally-active genes in N_2 fixing cultures or in those developing akinetes (Campbell et al. 2007). A majority of the up-regulated genes encode proteins for signal transduction and transcriptional regulation, with additional ones including genes encoding putative chemotaxis proteins and genes involved in pilus biogenesis (Meeks et al. 2001; Klint et al. 2006; Campbell et al. 2007). The genome of *N. punctiforme* has 3–5 copies each of the genes encoding homologues of the chemotaxis-related proteins CheA, CheB, CheW, CheD and CheR (Meeks et al. 2001). This chemotactic ability is probably particularly important in plants such as *Gunnera* and the cycads in which the symbiotic tissue is in dark locations and the hormogonia must override their natural positive phototaxis.

Chemotaxis is clearly important in the *Blasia*-cyanobacteria symbiosis because the N starved liverwort releases a very effective chemoattractant (Adams and Duggan 2008), although this is unlikely to be specific to *Blasia* as non-host plants such as *Trifolium repens* (Nilsson et al. 2006) and germinating wheat seeds (Adams and Duggan 2008) can also release hormogonia chemoattractants. No chemical identification of any of these chemoattractants has been made, although it has been suggested that they may be sugar-

based molecules; indeed, simple sugars such as arabinose, glucose and galactose do attract hormogonia, with arabinose being the most effective (Nilsson et al. 2006). Arabinose is found at high levels in *Gunnera* stem gland mucilage, possibly released from arabinogalactan proteins or arabinan-containing pectins by the extracellular enzyme ARAf, the gene for which is expressed at higher levels in stem tissue containing glands compared with that lacking glands (Khamar et al. 2010). The presence of high levels of arabinose in the mucilage ensures that hormogonia are attracted into the gland channels, and from there into the deeper tissues. Inside the mature gland tissue high levels of the reducing sugars glucose, fructose and sucrose suppress further hormogonia formation (Khamar et al. 2010).

It might be thought that hormogonia and chemotaxis are not needed in the *Azolla* symbiosis because the fern does not require *de novo* infection, yet filaments resembling hormogonia are found at the apical regions of the plant (Zheng et al. 2009a; Sect. 23.5.4). This hormogonia-like phase is without doubt important in the *Azolla* symbiosis as it has been retained during a long evolutionary history of association with the plant. These filaments are motile and possibly guided to the symbiotic cavity and the megasporocarps (Sect. 23.5.4) by chemotaxis (Zheng et al. 2009b). In support of this, preliminary annotation of the draft genome sequence of the obligate cyanobiont in the *A. filiculoides* symbiosis (http://genome.jgi-psf.org/anaaz/anaaz.home.html) reveals a substantial number of genes with potential involvement in signal perception and transduction of that signal into a developmental or behavioural response, as well as genes that may encode a pilus-related motility apparatus.

23.4.1.5 Other Characteristics

Apart from motility and chemotaxis there must be additional, more subtle characteristics of hormogonia that influence plant infection. Mutations in *cyaC*, which encodes adenylate cyclase, the enzyme responsible for the biosynthesis of the intracellular messenger adenosine 3′, 5′-cyclic monophosphate (cAMP), alter the efficiency of infection of *Blasia* by *Nostoc punctiforme*, implying that cAMP may play a role in infection (Adams and Duggan 2008; Chapman et al. 2008). However, the situation is complex because mutations in two different domains of the multi-domain CyaC adenylate cyclase result in different infection phenotypes in *Blasia*, with one mutant infecting at 25% of the wild-type frequency, but the other at 300–400% of the wild-type, even though both possess similar cellular cAMP levels at 25% of the wild-type (Chapman et al. 2008). There are no differences between the two mutants in terms of the frequency of hormogonia produced in the presence of *Blasia*, or the motility or piliation of the hormogonia. These data imply that cAMP *per se* is not involved in symbiotic competency, and that the contrasting infection phenotypes of the mutants are a result of unknown behavioural differences of the mutant hormogonia in response to plant signals.

Further evidence that the behaviour of hormogonia, as much as their abundance, influences host infection comes from a mutant of *Nostoc punctiforme* inactivated in the gene *sigH*, which encodes an alternative sigma subunit of RNA polymerase (Meeks 2003). Transcription of this gene is induced by a hormogonia-inducing factor (HIF; Sect. 23.4.2) from *Anthoceros*, yet a mutant inactivated in *sigH* produces the same frequency of HIF-induced hormogonia as the wild-type, although they are up to fivefold more infective of the hornwort than wild-type hormogonia (Meeks and Elhai 2002; Meeks 2003).

23.4.2 Signalling Between Potential Partners

It is of clear benefit to a plant host to stimulate hormogonia production in potential cyanobionts and so improve the chances of infection. To this end plants, particularly when starved of combined nitrogen, release factors that trigger the formation of hormogonia (hormogonia-inducing factor, HIF; see below) and act as chemoattractants. To improve their chances of infection host plants also produce a factor that prolongs the motile hormogonial phase. For example, a component of *Gunnera* stem gland mucilage increases the hormogonial stage from 20 to 40 h to several weeks (see: Bergman et al. 2007a). However, once inside a symbiotic cavity the plant releases a hormogonia-repressing factor (HRF) which is dominant over HIF and ensures that vegetative growth, heterocyst production and N_2 fixation are resumed (Sect. 23.6.1). Hormogonia-inducing factors have been found in the hornwort *Anthoceros punctatus* (Meeks et al. 2002; Meeks and Elhai 2002; Meeks 2003, 2009), cycads and the angiosperm *Gunnera* (Rai et al. 2000; Bergman et al. 2008a) and the liverwort *Blasia* (Watts 2000; Adams 2002a, b; Adams and Duggan 2008).

The chemical identity of HIF is not known, but available evidence implies that is will be different in different plants. The HIF of *Anthoceros punctatus* is a small, heat-labile compound released during N starvation (Meeks and Elhai 2002; Meeks 2003, 2009). Mutants of *Nostoc punctiforme* showing increased responsiveness to *Anthoceros* HIF also show a greater initial frequency of infection of the hornwort than the wild-type. The frequency of HIF-induced hormogonia in *N. punctiforme* is reduced by mutation of the gene *ntcA*, encoding the global transcriptional regulator NtcA (Herrero et al. 2004) and the resulting hormogonia fail to infect *Anthoceros* (Wong and Meeks 2002).

In the *Gunnera* symbiosis the structure of the gland, the presence of stipulate tissue and the presence of exuded mucus are all accommodations by the plant to encourage the presence of *Nostoc* hormogonia. However, the plant places a strong selection on the invading cyanobacteria and it is likely that a

system of positive and negative controls ensures that only cyanobacteria of the genus *Nostoc* are found within the stem glands. Evidence of the influence that *Gunnera* exerts on cyanobiont physiology is seen in *Nostoc* protein changes induced by the plant. Enhanced expression of three genes (*hieA*, *hieB* and *hieC*) in symbiotically-competent *Nostoc* is stimulated by *Gunnera* gland mucilage (Liaimer et al. 2001). The genes encode an outer membrane glycoprotein, a potential signalling compound and a protein that may be involved in adaptation of *Nostoc* to an acidic environment such as that in *Gunnera* mucilage (Liaimer et al. 2001; Bergman et al. 2007a). More recently, proteomic analysis has identified 38 proteins differentially expressed in symbiotic *Nostoc* cells compared with free-living cells (Ekman et al. 2006). Four are associated with the cell surface and are upregulated in the symbiotic organism; one of the four contains fasciclin-like repeats which have been implicated in symbiotically-important proteins in a lichen symbiosis (Paulsrud and Lindblad 2002).

Once *Nostoc* cells are within the *Gunnera* gland larger molecules may be responsible for signalling: either diffusible soluble molecules or larger, non-diffusible molecules found within the extracellular slime of the cyanobacterium or attached to the cell surfaces of either the cyanobacterium or the plant cells. Potential signalling compounds are the arabinogalactan proteins (AGPs) which are a very diverse group of proteoglycans generally found associated with the extracellular matrix of plant and some algal cells. Their structure, function and expression patterns have been well reviewed elsewhere (Seifert and Roberts 2007). AGPs have been associated with the initiation (and potentially the maintenance) of the *Alnus-Frankia* symbiosis, being found at the symbiotic interface in the root nodules (Berry et al. 2002). Early evidence has shown AGPs to be associated with *Gunnera* gland mucilage (see: Bergman 2002; Bergman et al. 2007a), but their role in the formation and maintenance of the *Gunnera-Nostoc* symbiosis has never been extensively investigated.

23.4.3 Other Important Factors

23.4.3.1 Lectins

The fungal partner of lichens and the plant host in bryophyte and *Azolla* symbioses produce lectins that can recognise and bind to sugars on the surface of symbiotic *Nostoc* strains (Lehr et al. 2000; see also: Rikkinen 2002; Adams et al. 2006). The cyanolichens *Peltigera canina* and *Leptogium corniculatum* produce an arginase that acts as a lectin by binding to a polygalactosylated urease in the *Nostoc* cell wall (Diaz et al. 2009; Vivas et al. 2010). Such lectins could be involved in fungus-partner recognition in lichens (Lehr et al. 2000; Elifio et al. 2000; Legaz et al. 2004; Sacristán et al. 2006), although proof of this is lacking; a model for the signalling pathways that might be involved has been proposed by Rikkinen (2002). In the *Geosiphon* symbiosis the only stage of the *Nostoc* life cycle that is incorporated by the fungus is the immotile primordial stage, which occurs immediately after the motile hormogonia stage (Sect. 23.5.6). Primordia are labelled by a mannose-specific lectin ConA, whereas heterocysts and hormogonia have different lectin-binding patterns (Kluge et al. 2002; Adams et al. 2006). This implies that alterations in *Nostoc* extracellular glycoconjugates could be important in recognition.

23.4.3.2 Proteins

Several proteins have been identified in the *Nostoc* proteome as being potentially important in symbiosis, but no temporal or spatial location has yet been attributed to them within a symbiotic system. One appears to be a two-component signal transduction system based on two putative proteins found in the proteome of diazotrophically-grown *Nostoc* PCC. 73102 (Ran et al. 2007). Another pair of proteins identified in the same study, a polysaccharide biosynthesis/export protein and a phosphomannomutase, were also suggested to be symbiotically important, although their exact roles remain to be elucidated. Another study identified a protein with 84% amino acid homology to a cyclodextrin glucosyltransferase, and hence the gene encoding the protein was named *cgt* (Wouters et al. 2003). These authors proposed several possible roles for Cgt in the fixation of N_2 under heterotrophic conditions and in the synthesis of the gelatinous material that coats filaments of *Nostoc* vegetative cells.

23.5 Host Structures and Their Infection

With the exception of the fungal associations (lichens and *Geosiphon pyriformis*) and to some extent *Gunnera*, cyanobacteria occupy existing structures in the host. Although cyanobacteria are photoautotrophs, many are also facultative photo- or chemoheterotrophs, which enables them to occupy locations that receive little or no light.

23.5.1 Hornworts and Liverworts

Bryophyte symbiotic structures are present in uninfected plants and so are not unique to the symbiotic state. In the liverwort *Blasia* the cyanobacteria occupy hemispherical structures known as auricles, on the underside of the thallus (Fig. 23.2c, d), whereas in hornworts such as *Anthoceros* and *Phaeoceros* they are found within the thallus in slime (mucilage) cavities (Fig. 23.2a) connected to the ventral surface of the thallus via mucilage clefts (Meeks 2003; Adams et al. 2006; Bergman et al. 2008a). Although mucilage clefts superficially resemble stomata, they are not thought to be related (Villarreal and Renzaglia 2006). The clefts form by

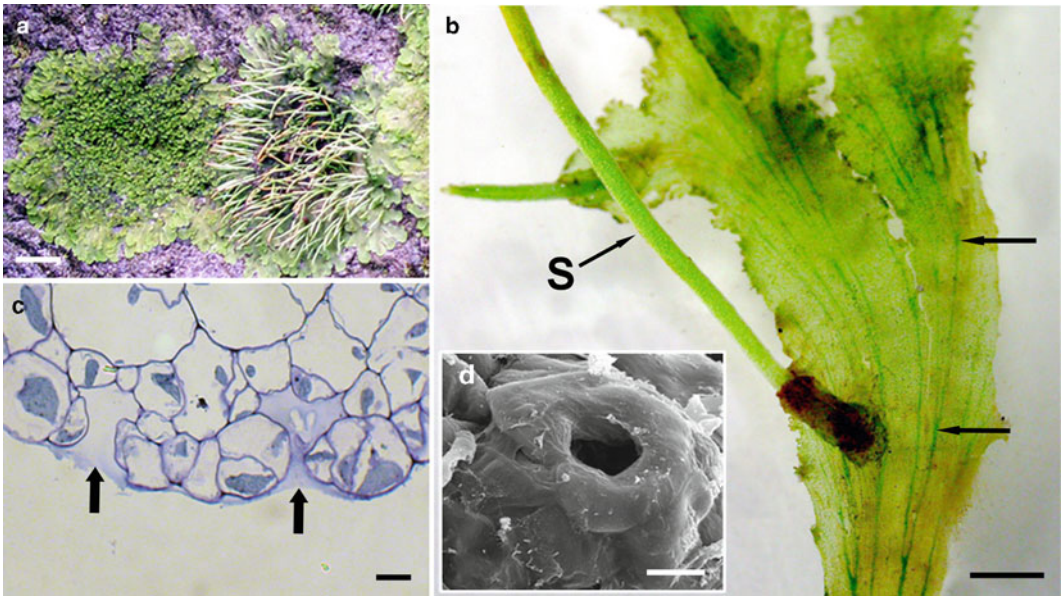

Fig. 23.19 The hornwort *Leiosporoceros dussii* with symbiotic *Nostoc*. (**a**) To the left is a young rosette and to the right an older thallus with numerous upright sporophytes. (**b**) Dark green *Nostoc* 'strands' (arrows) can be seen within the thallus, parallel to the main axis. *S* sporophyte. (**c**) Light micrograph of a nearly transverse section of two mucilage clefts (arrows), which provide the entry point for cyanobacterial infection; the *Nostoc* filaments subsequently spread through channels created by the separation of 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**). (Reproduced with permission from Villarreal and Renzaglia 2006)

apparently random separation of adjacent epidermal cells on the ventral side of the thallus; the slime cavity then develops beneath the cleft (Renzaglia et al. 2000). The *Blasia* auricle develops from a three-celled mucilage hair which undergoes extensive elaboration to form the dome-shaped auricle, which then becomes infected by *Nostoc* (Renzaglia et al. 2000). In the hornwort *Leiosporoceros dussii* (Fig. 23.19a) the cyanobacteria occupy mucilage-filled 'canals' (Fig. 23.19b) formed by the separation of plant cell walls along their middle lamellae (Villarreal and Renzaglia 2006). These canals run parallel to the main axis of the thallus and as they elongate they bifurcate to form an integrated network, enabling the *Nostoc* to spread internally throughout the thallus (Villarreal and Renzaglia 2006). By contrast, the isolated nature of the discrete colonies in *Blasia*, *Anthoceros* and *Phaeoceros*, means that each individual symbiotic structure must become infected from the outside.

It is motile hormogonia that enter hornwort slime cavities and the auricles of *Blasia*, but once inside the plant the priority is to establish a N_2-fixing colony, so motility is lost and heterocyst development is initiated (Adams 2002a, b; Adams et al. 2006). Entry to the hornwort slime cavities is through the mucilage clefts (Figs 23.2a and 23.19c, d) and there are interesting parallels between this and the likely infection route in a possible symbiotic relationship between the primitive, extinct land plant *Aglaophyton major* and an *Archaeothrix*-type filamentous cyanobacterium (Taylor and Krings 2005). From fossil evidence, cyanobacteria are thought to have entered the plant via stomatal pores, colonising the substomatal chambers and from there spreading throughout the outer cortical tissue, where they can be seen in fossil specimens. This hypothetical infection process is similar to that in the living hornwort *Leiosporoceros dussii* described above (Villarreal and Renzaglia 2006).

Although the establishment of a functional, N_2 fixing symbiosis requires the development of heterocysts, mutants incapable of heterocyst development are still able to infect *Anthoceros punctatus* (Wong and Meeks 2002). For example, strains inactivated in *hetR* (Wong and Meeks 2002), the primary driver of heterocyst differentiation (Golden and Yoon 2003; Zhang et al. 2006), and *hetF* (Wong and Meeks 2002), which is involved in the regulation of *hetR* transcription and the localisation of HetR to developing heterocysts (Wong and Meeks 2001), infect the hornwort as efficiently as the wild-type although they are incapable of supporting growth of the plant because they lack heterocysts (Wong and Meeks 2002). By contrast, inactivation of *ntcA*, which encodes the global nitrogen regulator NtcA (Flores and Herrero 2005), completely prevents infection even though the mutant produces motile hormogonia (Wong and Meeks 2002).

23.5.2 Cycads

It is the coralloid roots of cycads that house their cyanobionts (Costa and Lindblad 2002; Lindblad and Costa 2002;

Bergman et al. 2007a, 2008a). These roots constitute approximately 1–3.6% of plant biomass and display negative geotropism, growing outwards and upwards from the tap root, sometimes breaking the surface of the soil. The cyanobionts are found in a mucilage-filled zone between the inner and outer cortical layers (Fig. 23.3). The formation of coralloid roots occurs in plants in the absence of cyanobacteria and so is not a response to infection. However, entry of the cyanobacteria into the coralloids does trigger significant morphological change, including an increase in root diameter and elongation of the cells linking the inner and outer cortical layers, possibly to facilitate nutrient exchange (Costa and Lindblad 2002). How the cyanobacteria gain entry to the coralloid root is unclear, but possible points of entry include lenticels, or breaks in the dermal layer (Costa and Lindblad 2002; Bergman et al. 2007a) and it has been suggested that 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).

23.5.3 *Gunnera*

In *Gunnera* the site of entry for potential cyanobionts is glands found at the base of each leaf stem (petiole) and covered in red tissue and a sticky mucilage (Fig. 23.5). The mucilage is secreted in large amounts by glands in mature plants and plays vital roles in the symbiosis, including the induction and chemoattraction of hormogonia (Bergman 2002; Bergman et al. 2007a). In larger species of *Gunnera* these glands are surrounded by 'stipulate' tissue, made up of long leaf-like fronds (Fig. 23.4), and it has been suggested that this tissue type is required to allow *Nostoc* access to the plant glands, which may be several metres above the soil (Benson and Margulis 2002). The gland itself is made up of 6–9 outward-facing papillae, one forming a central stem with the others surrounding it (Fig. 23.5b). These glands are seen in all *Gunnera* plants, including those grown under sterile conditions and not brought into contact with any cyanobacteria. Formation of the glands appears to be triggered by low environmental N and hence their formation is related to the plant's requirement for fixed nitrogen (Chiu et al. 2005). Furthermore, the structural features of the glands appear vital to the formation of the symbiosis because removal of the outlying papillae, leaving only the central structure, prevents the plant from forming a stable symbiosis. However, only one of the outer papillae is needed to re-establish the capacity for symbiosis (Uheda and Silvester 2001). This suggests that the cyanobacteria travel down the channels between the papillae (Fig. 23.6a, b) to the bottom of the glands, and the papillial structures have an essential role in allowing the cyanobacteria to invade the cells at the base of the gland (Fig. 23.6c, d).

Once the hormogonia have reached the interior of the *Gunnera* stem gland they invade the plant cells by an undetermined mechanism (Bergman 2002; Bergman et al. 2007a). Localised mitotic activity near the infection site might be caused by the phytohormone indole-3-acetic acid (IAA) which symbiotic cyanobacteria are capable of producing (Sergeeva et al. 2002). It is thought that these dividing cells are the ones that become infected (Bergman et al. 2007a). Once cell invasion has taken place, the cell wall appears normal in host cells containing the cyanobionts. At this stage the gland channels disappear, preventing any further infection. Delineated clusters of cells containing the cyanobiont appear, and are bordered by layers of uninfected plant cells. The formation of such "organs" is unusual, and may suggest further extracellular signalling between the plant and the cyanobacterium. Within the *Gunnera* cells, the cyanobacteria form clusters and fill the space, although they never enter the cell cytoplasm as they never penetrate the plant cell plasmalemma, which is thought to act as the interface between the plant and its cyanobiont, where all nutrients (travelling both ways) are exchanged (Bergman 2002; Bergman et al. 2007a).

23.5.4 *Azolla*

Azolla has overlapping leaves consisting of two lobes each approximately 1 mm in length (Fig. 23.7a, b), the thick, aerial dorsal lobe containing chlorophyll and the partially-submerged, thinner achlorophyllous ventral lobe serving as a float. Dorsal lobes have an extracellular ovoid cavity, approximately 0.3 mm in length, formed by an infolding of the adaxial epidermis during development (van Hove and Lejeune 2002a; Lechno-Yossef and Nierzwicki-Bauer 2002; Bergman et al. 2007a, b; Zheng et al. 2009a). The cavity, which is at first open to the outside, becomes closed as the leaf matures, and is occupied by a highly complex prokaryotic community, including 2,000–5,000 cyanobiont cells and numerous heterotrophic bacteria (bactobionts or eubionts), that remains intimately associated with the plant throughout its life cycle. In mature leaf cavities the bactobionts and cyanobacteria are found at the periphery, immobilised within a polysaccharide-rich mucilaginous material between internal and external envelopes, which are probably of plant origin and function in metabolite exchange (van Hove and Lejeune 2002a, b; Lechno-Yossef and Nierzwicki-Bauer 2002). The central region of the cavity is free of symbionts and is gas-filled (Lechno-Yossef and Nierzwicki-Bauer 2002). A pore in the adaxial epidermis of the leaf cavity connects it with the external environment and probably functions in gas exchange. The pore is lined with teat-shaped cells which may function in water repulsion and serve as a physical barrier, blocking the entry of particles and organisms and preventing the cyanobacteria bactobionts from leaving (Veys et al. 2000, 2002; see also Zheng et al. 2009a).

As many as 25 simple hairs, together with two primary branched hairs, protrude into the mucilage layer around the periphery of the leaf cavity and function in metabolite/signal exchange between the host and its symbionts (Lechno-Yossef and Nierzwicki-Bauer 2002; Pereira and Carrapiço 2007; Zheng et al. 2009a). Indeed, both the cytoplasm and chloroplasts of branched hair cells contain higher glutamine synthetase levels than those in cyanobiont-free plants (Uheda et al. 2004), implying that these cells are involved in the assimilation of N released by the cyanobiont. Branched hairs are associated with the cavity throughout its development and facilitate the inoculation of developing leaf cavities with both cyanobacteria and bactobionts. By contrast, simple hairs increase in number as a cavity matures. The hairs are also present in cyanobiont-free cavities and are fundamental to the maintenance of the *Azolla*-cyanobacteria association; their role, along with other structural features of this association, has been reviewed recently (Zheng et al. 2009a).

The permanent association of *Azolla* and its cyanobiont is possible because the fern's reproduction processes are inextricably linked with transfer of cyanobacteria to each new plant generation. *Azolla* is able to reproduce both sexually (a complex process involving sporocarp production) and asexually (the main form of reproduction). Asexual reproduction is rapid, with a doubling time of 2 days or less under optimal laboratory conditions and involves fragmentation of branches from the main plant stem (rhizome). The apical regions of *Azolla* contain rapidly-dividing undifferentiated cyanobacterial filaments resembling hormogonia, which are introduced into the leaf primordium before development of the leaf and new leaf cavity are complete (Zheng et al. 2009b). Primary branched hairs form bridge-like structures at the apical meristems, promoting the partitioning of the cyanobacterial inoculum into the young leaf cavities (Zheng et al. 2009a, b). Sexual reproduction in *Azolla* is less common and appears to be triggered by adverse environmental conditions, plant density and light intensity (reviewed by Lechno-Yossef and Nierzwicki-Bauer 2002; Pabby et al. 2004a). *Azolla* sporophytes produce two morphologically-distinct sporocarps, the male microsporocarps and the female megasporocarps. The latter contain a single megasporangium (consisting of a single megaspore and the megaspore apparatus) and a colony of *Anabaena*.

The process by which *Anabaena* is packaged into the developing megasporocarp pairs, thereby securing its horizontal transfer to the next plant generation, has attracted considerable interest. Zheng and co-workers (2009b) have examined the development of entrapped cyanobacteria in the developing megasporocarps of *A. microphylla* fronds located at the second, fourth and fifth branch points (approximately 5, 10 and 17 days old, respectively). A population of small-celled motile cyanobacterial filaments resembling hormogonia enter the developing sporocarps through a pore at the top of the indusium chamber (Fig. 23.7e), presumably guided by chemotaxis towards the developing megasporocarp pairs found in the apical region of the sporophyte (Zheng et al. 2009b). Following entry of the hormogonia into the developing megasporocarp, the cells of the filaments undergo synchronous conversion to spore-like resting cells known as akinetes (Fig. 23.8; Zheng et al. 2009b), with the result that the cyanobacterial population in the mature megasporocarp consists primarily of large, individual akinetes (Figs. 23.7e and 23.8f). One of the triggers for akinete development in free-living cyanobacteria is phosphorus limitation and this may be the trigger in the megasporocarp, as polyphosphate granules are rarely observed in the cyanobacteria during their transfer to the megasporocarp (Zheng et al. 2009b), possibly as a result of impaired phosphate uptake (Ran et al. 2010). The megasporocarp pore closes following the colonization phase, thereby preventing further entry of cyanobacteria and the other bacteria that are associated with the hormogonia-like filaments in the apical region (Zheng et al. 2009b). Given the status of the akinete as a survival structure it is not surprising that the mature megasporocarp, awaiting germination, harbours the cyanobiont in its own resting phase. This represents an example of the synchronous development that occurs between the cyanobiont and its host, a strategy that has evolved to maintain this unique plant-cyanobacterial association. Similarly, fertilisation and embryogenesis are followed by akinete germination to produce metabolically-active vegetative filaments which, with the assistance of the cotyledonary hairs, are introduced into the embryonic leaf (Zheng et al. 2009a).

23.5.5 Lichens

Unlike any other cyanobacterial symbiosis the morphology of lichens (Fig. 23.9) bears no resemblance to that of the free-living partners, and although the cyanobiont can influence thallus development, it is mostly the mycobiont that determines the morphology and chemistry of the lichen (Rai and Bergman 2002; Rikkinen 2002; Sanders 2006). So, the fungal thallus that will house the photobionts exists only in the symbiotic state. Lichens generally, although not exclusively, fall into three morphological classes referred to as crustose (a thin, crust-like layer), foliose (leaf-like) and fruticose (branched). In bipartite cyanolichens the cyanobacterium generally forms a continuous layer beneath the upper cortex (Fig. 23.10d), but can be dispersed throughout the thallus (Fig. 23.10b, c). In the tripartite lichens, the green algal photobiont occurs as a layer throughout the thallus, but the cyanobacterium is isolated in specialised structures known as cephalodia, either within the thallus or on its surface (Fig. 23.10a; Rikkinen 2002; Adams et al. 2006). Cephalodia only form when cyanobacteria are present

and the formation of each is a new event (Rai et al. 2000). These structural arrangements are not always so simple; for example in chimeroid lichens, known as photosymbiodemes, green algae and cyanobacteria are the primary photobionts in different parts of the thallus, with a gradual transition between the two, often along a light or humidity gradient (Rikkinen 2002). By contrast, the 'jelly lichens' of the Collemataceae produce homoiomerous thalli, lacking a distinct photobiont layer, and owe their gelatinous habit to the copious extracellular polysaccharide produced by the *Nostoc* cyanobionts (Wedin et al. 2009).

23.5.6 *Geosiphon pyriformis*

In *Geosiphon pyriformis* the specialised fungal structures (bladders; Fig. 23.11b, c) that house the cyanobionts are only produced in response to the presence of suitable cyanobacteria. The *Nostoc* cyanobiont is intracellular, and is incorporated into the fungal hyphae by endocytosis (Kluge 2002; Kluge et al. 2002; Adams et al. 2006). The *Nostoc* must be at the "primordium" stage (Sect. 23.4.1.2) of its life cycle to be recognised by the fungus; primordia are formed when motile hormogonia lose their motility and produce the first heterocysts. The process of incorporation begins when the tips of the fungal hyphae encounter *Nostoc* primordia and the fungal wall softens and bulges outwards, surrounding usually 5–15 *Nostoc* vegetative cells (Fig. 23.11a), although existing heterocysts are cut off and are not endocytosed (Kluge et al. 2002; Adams et al. 2006).

Each infected hypha swells to form a 2 mm long, pear-shaped multinucleate bladder, that is coenocytic with the fungal mycelium and contains the cyanobacteria (Fig. 23.11b, c). Bladders without endosymbionts are never found, implying that formation of the bladder is a specific response to the endocytosis of the cyanobacteria. The bladders are highly vacuolated and at the basal end, which is beneath the soil surface, are milky white in appearance due to the presence of numerous lipid droplets (Fig. 23.11c). The apical three quarters of the bladder is above the soil surface and is dark in appearance due to the presence of the *Nostoc* symbionts (Adams et al. 2006). The symbionts occupy a cup-shaped compartment known as the symbiosome, formed by invagination of the fungal plasma membrane (Fig. 23.11c). The symbiotic interface is the space between the host membrane and the *Nostoc* cell wall and this is filled with chitin, resembling the symbiotic interface between the fungal wall and the plant plasma membrane in arbuscular mycorrhizas (Adams et al. 2006). Heterocysts are present in the *Nostoc* within the bladders, although their frequency is not elevated as it is in many cyanobacterium-plant symbioses (Sect. 23.6.2.2) and this reflects the primary role of the cyanobiont in *Geosiphon*, which is to provide fixed carbon for the fungus (Kluge 2002).

23.5.7 Diatoms and Dinoflagellates

In the symbiosis between the diatom *Rhizosolenia* and the heterocystous cyanobacterium *Richelia intracellularis* the cyanobiont is found extracellularly in the host's periplasmic space, between the plasmalemma and the frustule (Carpenter 2002; Bergman et al. 2007a). By contrast, in *Rhopalodia* the unicellular cyanobiont is located in the cytoplasm but separated by a host membrane (Rai et al. 2000; Janson 2002; Bergman et al. 2007a).

In the dinoflagellate-cyanobacteria symbioses the cyanobiont location varies in the different genera of dinoflagellates (Fig. 23.14; Carpenter 2002); in *Ornithocercus*, *Citharistes*, *Histioneis* and *Parahistioneis* the cyanobacteria are located externally to the host cytoplasm, whereas in *Amphisolenia* they are within the cytoplasm. In *Ornithocercus* the cyanobionts are located between the upper and lower cingular list, whereas in *Histioneis* they are in a chamber on the girdle floor (Jyothibabu et al. 2006).

23.5.8 Sponges

Sponges vary in size from less than 1 cm to several metres and are sessile filter feeders which collect food from the large volumes of water that pass through an aquiferous canal system. The sponge body is hollow, the wall consisting of a layer of pinacocyte cells on the outside and a layer of choanocytes on the inside, separated by the gelatinous mesohyl. It is the beating of flagella on the choanocytes that circulates water through the sponge. The supporting skeleton, consisting of a fibrous protein and spicules made of silica or calcium carbonate, is found in the mesohyl. In larger sponges the wall becomes pleated and the mesohyl is thickened and embedded with many interconnected choanocyte chambers. The inner part of the sponge is known as the endosome (or choanosome) and this is protected from strong currents and high light intensities by the outer layers. Food particles are taken up by phagocytosis in the choanocyte chambers located in the endosome.

Symbiotic cyanobacteria are usually intercellular, but sometimes occur in specialised vacuoles called cyanocytes (Usher 2008). In general, cyanobacteria are found in the outermost few millimetres of the sponge, where light availability is greatest, whereas the internal mesohyl contains a complex mixture of symbiotic heterotrophic and autotrophic bacteria (Hentschel et al. 2006). However, there are exceptions such as *Oscillatoria spongeliae* found abundantly in the mesohyl, but not in the surface layers of the sponge *Lendenfeldia chondrodes* (Ridley et al. 2005b), and *Synechococcus* present in the endosome of *Tethya aurantium* (Thiel et al. 2007). In the latter example it seems

that radiating silica spicules act as a fibre-optic system to conduct light into the deeper sponge tissue (Brümmer et al. 2008).

23.5.9 Ascidians

In the cyanobacteria-ascidian symbioses the cyanobiont can be found on the colony surface, in the peribranchial and common cloacal cavity (Hirose and Hirose 2007; Hirose et al. 2009a, b; Kojima and Hirose 2010), or in the tunic (Hirose et al. 2006b; Hirose and Nakabayashi 2008), depending on the host species. In the case of *Trididemnum clinides* the three cyanobionts differ in their distribution in the host tunic, type-A (possibly a novel non-*Prochloron* species) being found predominantly near the colony surface, together with small numbers of type-C (a possible *Oscillatoria*), whereas type-B (possibly *Synechocystis didemni*) is found throughout the tunic (Hirose et al. 2009b). This segregated distribution is thought to result from the cyanobionts occupying the location most suited to their different pigment contents.

Cyanobionts can be either intracellular or extracellular depending on the didemnid and on the cyanobiont location within the animal. For example, in *Lissoclinum punctatum* the *Prochloron* cells found in the tunic are mostly intracellular, whereas those in the peribranchial and common cloacal activities are extracellular (Kojima and Hirose 2010). *Prochloron* cells attach to the tunic wall because of the adhesive nature of the wall lining (Hirose and Fukuda 2006; Hirose and Nakabayashi 2008). The tunic of photosymbiotic didemnids is usually transparent, although they do contain UV-absorbing substances (such as mycosporine-like amino acids, MAAs), calcareous spicules and pigmented tunic cells (Hirose et al. 2004; Hirabayashi et al. 2006) which may create an ideal light environment for the cyanobionts (Hirose et al. 2009b). For example, *Didemnum molle* colonies in shallow water (10 m) have high MAA concentration and low spicule density to block UV without attenuating photosynthetically-active radiation, whereas colonies in deeper water (20 m) have much lower concentrations of MAAs (Hirose et al. 2006a).

23.6 Host-Cyanobiont Interactions Post-infection

Cyanobacteria in symbiosis, particularly with plants, frequently show morphological and physiological modifications including repression of hormogonia development, increased cell size, reduced growth rate, increased heterocyst frequency and N_2 fixation, and depressed N assimilation and carbon dioxide fixation.

23.6.1 Hormogonia Repression and Cell Division Control

23.6.1.1 Hormogonia Repression

Although it is to a host plant's advantage to induce hormogonia formation in potential symbionts, once the cyanobacterium has infected the plant it is essential that hormogonia formation is repressed, to facilitate heterocyst development and N_2 fixation. To this end a water-soluble hormogonium repressing factor (HRF), which is dominant over the hormogonia-inducing HIF signal, is released into the symbiotic cavity in the hornwort *Anthoceros punctatus* (and presumably also in liverworts and *Gunnera*) to inhibit hormogonia formation even in the presence of HIF (Meeks and Elhai 2002; Meeks et al. 2002; Meeks 2003). In *Nostoc punctiforme* HRF induces expression of *hrmA*, which has no significant sequence homology with genes in the databases but which is part of a *hrmRIUA* operon that is similar in sequence to sugar uronate metabolism operons of other bacteria (Campbell et al. 2003; Meeks 2003). The expression of *hrmA* is also induced by the plant flavonoid naringin (Cohen and Yamasaki 2000). A strain mutated in *hrmA* forms hormogonia in the presence of HRF. Hormogonia repression is achieved through the sugar-binding transcriptional repressor HrmR, which prevents any new rounds of development (Campbell et al. 2003). There is evidence that fructose (possibly converted to a signalling metabolite) may be involved in the repression of hormogonia formation (Ungerer et al. 2008). Indeed, hormogonia formation is repressed by sucrose, glucose and fructose, the latter two of which are present at high concentrations in *Gunnera manicata* mature stem gland tissue, repressing further hormogonia formation and thereby enabling heterocyst differentiation and N_2 fixation (Khamar et al. 2010).

The *hrm* operon may be involved in symbiotic systems other than *Anthoceros* because aqueous extracts from fronds of *Azolla pinnata* and *A. filiculoides* are potent inducers of *hrmA* expression in *N. punctiforme* strain UCD 328 (Cohen et al. 2002). Moreover deoxyanthocyanin, the coloured pigment in *Azolla* leaves that increases during the winter months and under phosphate limitation, producing a distinct reddish hue, acts in synergy when mixed with other plant-derived compounds, resulting in a significant increase in *hrmA* induction (Cohen et al. 2002). Mature *Azolla* frond tissue is redder than actively-growing apical regions which may imply a role for deoxyanthocyanins as an HRF component.

23.6.1.2 Cell Division Control

In many cyanobacterial symbioses, particularly those involving plants, the host grows much more slowly than its symbiont. Therefore, to maintain a stable symbiosis and avoid being rapidly out-grown the host must regulate the growth of the cyanobiont. Strategies include the blocking of cell division,

physical confinement (by restricting the number and size of the symbiotic structures) and restriction of the nutrient supply (Rai et al. 2000; Ekman et al. 2006; Bergman et al. 2007a). In the case of the hornwort *Anthoceros* the growth rate of symbiotically-associated *Nostoc* can be up to tenfold slower than when in the free-living state (Meeks 2003). Although the mechanism is not known, *Anthoceros* can also regulate *Nostoc* colony biomass and N_2 fixation rate to ensure that the rate of N_2 fixation per unit of plant tissue remains constant when, for example, the formation of new colonies is inhibited by penicillin, or growth is stimulated at elevated light intensity and CO_2 (Meeks and Elhai 2002; Meeks 2003).

In *Azolla*, growth of the cyanobiont can be differentially regulated in specific regions of the plant. Growth rates of both partners in the *Azolla* association are at their maximum in the apical regions and decrease linearly along the axis away from the apex, towards the mature regions of the host (Bergman et al. 2007a, b). When plant growth is inhibited with cycloheximide (a specific inhibitor of protein synthesis in eukaryotes), the rapid cell division occurring in the cyanobiont present in the plant apex also stops. The cyanobiont population (number of cells per cavity) and cell size also increase from apical to older regions of the fern (leaf numbers 1–15). Although the cell size continues to increase in much older regions of the plant (leaf numbers 15–28), the population of the cyanobiont becomes constant (see: Bergman et al. 2007a, b). This regulation of growth may not apply so stringently to the primary *Azolla* cyanobiont, which is readily detected in cyanobacterial preparations from crushed whole plant tissues using molecular probes (see: Meeks 2009). By contrast, the secondary (and culturable) cyanobiont is not detected using the same approach, leading to the suggestion that these cyanobionts are present at a level significantly lower than that of the primary cyanobiont, and their growth is under stringent control by the host. The apparent lack of growth control over the primary cyanobiont might be a reflection of some of the adaptations that have evolved for life in obligate symbiosis (Meeks 2009).

Evidence of host control over growth and cell division of cyanobionts is also found in sponges in which cyanobacterial abundance seems to be proportional to the number of sponge cells (Taylor et al. 2007). How such control is effected isn't clear, although restriction of the cyanobiont's access to essential nutrients and the sequestration of carbon fixed by the cyanobiont are possible mechanisms (Taylor et al. 2007).

23.6.2 Morphological Modifications

23.6.2.1 Cell Morphology

Although the morphology of cyanobacterial symbionts in sponges seems not to be affected by the host or its biogeographic location (Usher et al. 2006), it is more common for the cell morphology of the cyanobacteria to be altered in symbiosis. For example, filamentous growth can become aseriate, and cells are often enlarged and altered in shape. Such changes often display an increasing gradient of severity from the youngest to the oldest symbiotic tissues. Cell enlargement and shape irregularity are apparent in the vegetative cells of *Nostoc* associated with hornworts (Meeks and Elhai 2002). In cyanolichens filamentous cyanobacteria such as *Scytonema* and *Calothrix* can become unicellular and *Nostoc* cell size increases (Rai and Bergman 2002). In the *Geosiphon* symbiosis, once the *Nostoc* cells are engulfed by the fungal hyphae they seem to undergo a period of stress, when they become deformed and their photosynthetic pigments become bleached (Fig. 23.11a; Kluge et al. 2002; Adams et al. 2006). Some cells die at this stage, but within 3 days the remainder recover and enlarge to approximately six times their free-living volume. *Nostoc* ultrastructure seems little changed inside *Geosiphon*, although the outer membrane is difficult to discern in electron micrographs and the heterocyst cell wall is thinner than usual, possibly indicating a reduced concentration of oxygen within the bladders (Adams et al. 2006).

The enlarged-cell phenotype is also seen in the *Azolla* leaf cavity where the volumes of both the vegetative cells and heterocysts of the cyanobiont are approximately four times greater than those of free-living cyanobacteria (see: Zheng et al. 2009a). Other morphological differences include vegetative cell shape, filament shape and pigmentation, and heterocyst cell shape and frequency (Pabby et al. 2003, 2004b; Papaefthimiou et al. 2008a; Sood et al. 2008a, b). The minor *Azolla* cyanobionts, which are able to grow under free-living conditions, retain the ability to utilise external supplies of sugar and show the modified morphological and physiological characteristics of symbiotic growth, including larger cells, increased frequency of heterocysts, increased N_2 fixation and increased respiration, leading to the suggestion that sugar metabolism rather than plant-derived factors may regulate some of the changes associated with symbiosis (Ungerer et al. 2008).

In general, the *Nostoc* cyanobionts of cycads show little morphological or structural change from free-living strains (Costa and Lindblad 2002), although there have been reports of cyanobionts with vegetative cells and heterocysts showing considerable degradation of the peptidoglycan layer (Baulina and Lobakova 2003a, b). However, it is not clear whether such cells are functional or are in various states of senescence.

23.6.2.2 Heterocyst Frequency

A characteristic of cyanobacterial symbioses involving a photosynthetic host is a significant increase in cyanobiont heterocyst frequency above that in the free-living state (Table 23.1), with a consequent enhancement of N_2 fixing capacity. There is often an increasing gradient of heterocyst

Table 23.1 Heterocyst frequency and the characteristics of glutamine synthetase in symbiotically-associated cyanobacteria. (Table compiled from Meeks (2009) and Adams (2000) and references therein)

Host	Heterocyst frequency (%)	Glutamine synthetase		Form of combined nitrogen released (% released)
		Amount of protein (%)	Specific activity (%)	
Cycads	17–46	100	100	Glutamine/citrulline? (ND)
Gunnera	20–60	100	70	NH_4^+ (90)
Hornworts and liverworts	25–45	86–100	~15	NH_4^+ (80)
Azolla	26–45	5–40	~30	NH_4^+ (40)
Lichens:				
Bipartite	4–8	<10	<10	NH_4^+ (90)
Tripartite	10–55	<10	<10	NH_4^+ (90)

Heterocyst frequency is expressed as a percentage of the sum of heterocysts plus vegetative cells. The range of frequencies reflects the increasing heterocyst frequency found with increasing age of symbiotic tissue. Typical frequencies for free-living cyanobacteria are 4–10%. Glutamine synthetase is expressed as the amount of protein (as a percentage of that in the same cyanobacterium growing in the free-living state) or the specific activity (as a percentage of that in the same cyanobacterium growing in the free-living state). The form of N released by the cyanobacterium to the host is given, with (in parenthesis) the amount of N released by the cyanobiont as a percentage of the total N_2 fixed. ND not determined

frequency from approximately 10–15% in the most actively growing region of the plant (such as the root tip in cycads) to 60% in old symbiotic tissue, although the highest rate of N_2 fixation often occurs at an intermediate heterocyst frequency. There is also a loss of symbiont CO_2-fixing capacity because of the reduction in vegetative cell frequency and the inability of heterocysts to fix CO_2, but this is compensated by a supply of fixed carbon from the host (or the green algal symbiont in the case of tripartite lichens) and a shift to a photo- or chemoheterotrophic mode of nutrition in the cyanobiont.

In the hornworts and liverworts cyanobionts have 6–10-fold higher heterocyst frequencies than in the free-living state (Table 23.1; Meeks 2003). Although some of these additional heterocysts may be non-functional, the elevated frequency nevertheless enhances the N_2 fixing capacity of the symbiotic colonies (Meeks and Elhai 2002; Meeks et al. 2002). Within the colonies heterocysts can be difficult to recognise because they often lose some of the morphological traits that distinguish them from vegetative cells, including their regular shape and thickened cell walls (Meeks and Elhai 2002; Adams and Duggan 2008).

In the *Azolla* cyanobiont heterocysts are absent in the very young leaves at the stem apex and first appear in filaments as they become enclosed within the symbiotic cavity in leaf 1. Heterocysts increase in frequency with successive leaves, reaching a maximum of 25–45% of the cell population by leaves 12–15 (Table 23.1; see: Lechno-Yossef and Nierzwicki-Bauer 2002; Pabby et al. 2004a). Despite the high heterocyst frequencies, double, triple or higher numbers of contiguous heterocysts are as infrequent as they are in free-living cultures (Meeks and Elhai 2002). Exogenous supplies of fixed nitrogen have limited inhibitory effects on heterocyst frequency and nitrogenase activity in the *Azolla* association (Meeks and Elhai 2002; Meeks 2009), raising the possibility that a plant-derived signal stimulates heterocyst development in symbiosis (see below). The cyanobionts in the coralloid roots of cycads also show a gradient of heterocyst frequency, the lowest (16.7%) being found at the growing root tip and the highest (46%; Table 23.1) in the older, basal tissue, where groups of up to four adjacent heterocysts can be found (Costa and Lindblad 2002).

In *Gunnera* glands *Nostoc* heterocyst frequencies of up to 60–80% are observed, whereas levels greater than 10% are not seen in free-living *Nostoc* cultures (Table 23.1; Bergman et al. 2007a; Franche et al. 2009). Nitrogen fixation activity increases in parallel with the increasing heterocyst frequency up to 20% heterocysts after which it declines, despite the continued increase in heterocyst frequency (Bergman 2002). The highest heterocyst frequencies are observed in the oldest symbiotic tissue, 10 mm or more from the stem apex, although *hetR*, the key gene in heterocyst development, is most highly expressed 7–8 mm from the apex (Wang et al. 2004).

In all tripartite lichens the cyanobiont role of N_2 fixation is aided by elevated heterocyst frequencies of 10–55% (Table 23.1) compared with 5–10% in free-living cyanobacteria (Rai 2002; Rai and Bergman 2002; Adams et al. 2006). Again, an increasing trend in heterocyst frequency is found from the youngest to the oldest parts of the thallus. By contrast, the role of the cyanobiont in two-membered cyanolichens is to provide both N and C, which means that elevated heterocyst frequencies (with the resulting decrease in carbon fixation capacity) are not sustainable, so frequencies are similar to those in free-living cyanobacteria (Table 23.1; Rai 2002; Rai and Bergman 2002; Bergman et al. 2007a). Theoretical models of tripartite lichens have confirmed that it is in the interest of the partnership to maximise heterocyst frequency and to maintain a low ratio of cyanobacterial cells to green algal cells (Hyvärinen et al. 2002).

What Regulates Heterocyst Frequency in Plants?

The accuracy of the heterocyst frequencies determined for plant-associated cyanobionts can be questioned because such heterocysts often lose their characteristic regular shape and thickened walls, making them difficult to distinguish from vegetative cells (Meeks and Elhai 2002; Meeks 2003, 2009). In addition, of those heterocysts that can be recognised by light or electron microscopy, at least some are likely to be senescent or dead (Meeks and Elhai 2002). This is supported by the observation that maximum N_2 fixation rates in cyanobionts *in planta* usually occur at intermediate heterocyst frequencies, implying that the multiple contiguous heterocysts found at the highest frequencies include heterocysts that are metabolically inactive. This low metabolic activity may be because they have either reached the end of their natural lifespan, or they are poorly supplied with photosynthate which has to pass from vegetative cells, through the outer heterocysts in a contiguous group, to reach those at the centre of the group. The presence of senescent heterocysts may also at least partly explain the development of multiple contiguous heterocysts, because a new heterocyst may develop next to an existing, but non-functional heterocyst (Meeks and Elhai 2002; Meeks 2009). However, such an explanation is unlikely to apply to the contiguous heterocysts formed in the *Gunnera* isolate *Nostoc* PCC9229 when grown on fructose in the dark (see below; Wouters et al. 2000).

Notwithstanding the above provisos, there is no doubt that heterocyst frequency is greatly elevated in plant symbioses. The question is – how is this controlled? In free-living cyanobacteria elevated heterocyst frequencies can be induced by the immobilisation of filaments in polyurethane or polyvinyl foams and other hollow matrices, or by short-term exposure to increased light intensity or the amino acid analogue 7-azatryptophan (Adams 2000). In the *Gunnera* isolate *Nostoc* PCC9229 three weeks of dark growth in the presence of fructose leads to the development of double and quadruple heterocysts, which are not formed in the absence of fructose (Wouters et al. 2000). These observations raise the possibility that the elevated heterocyst frequencies found in cyanobionts *in planta* may be at least in part due to the environmental conditions they experience.

The signal for heterocyst development in free-living cyanobacteria is starvation for combined nitrogen, which is thought to be perceived via an elevated intracellular concentration of 2-oxoglutarate (Muro-Pastor et al. 2001; Vazquez-Bermudez et al. 2002; Zhang et al. 2006). 2-oxoglutarate activates NtcA, a transcriptional regulator that controls the expression of genes encoding proteins for the uptake and metabolism of N sources other than ammonium (Flores and Herrero 2005; Muro-Pastor et al. 2005). NtcA in turn stimulates transcription of genes encoding both positive (e.g. HetR and HetF) and negative (e.g. PatN and PatS) regulators of heterocyst development (Herrero et al. 2004; Zhang et al. 2006; Meeks 2009).

However, starvation for combined nitrogen is unlikely to be the signal for heterocyst development in symbiotically-associated *Nostoc* because the cyanobiont does not show any of the characteristic features of N limitation, such as the breakdown of N reserves (Sect. 23.6.3). This may imply that the signal for heterocyst differentiation in symbiosis is supplied by the host plant. Support for this theory comes from the behaviour of a *Nostoc punctiforme* mutant defective in the assimilation of nitrate, which as a consequence fails to repress heterocyst development and N_2 fixation (Meeks and Elhai 2002; Meeks 2003). Heterocysts that form in the mutant in the presence of nitrate are defective and incapable of N_2 fixation under oxic conditions, but can fix N_2 within the anoxic slime cavities of the hornwort *Anthoceros*. However, within these slime cavities N_2 fixation is repressed by nitrate, unlike the situation in free-living cultures of the mutant. This repression was shown not to be due to a build-up of ammonium resulting from the reduction of nitrate by *Anthoceros*. These observations imply that *in planta* the regulation of N_2 fixation and heterocyst development is plant-mediated and independent of the N status of the cyanobiont (Meeks 2003, 2009). The molecular target(s) for this plant signal remains to be identified but it seems highly likely that it acts prior to activation of the key heterocyst differentiation gene *hetR* (Zhang et al. 2006) and also prior to *ntcA* (discussed further by Meeks and Elhai 2002; Wong and Meeks 2002; Meeks 2009).

23.6.2.3 Host Changes

Whereas morphological changes are often apparent in symbiotically-associated cyanobacteria, there are generally few obvious post-infection changes in the host. For example, in the *Azolla* association the leaf cavity and the hairs (which are fundamental to the functioning of the association with the cyanobionts) also exist in cyanobiont-free *Azolla* (see: Zheng et al. 2009a), although mucilage production (possibly by all partners in the association) is often regarded as a symbiosis-related change. Those morphological changes that are apparent in hosts often reflect the need for efficient nutrient exchange between the partners, which is essential for a stable symbiosis. Such changes are seen in both *Blasia* and *Anthoceros*; branched, multicellular filaments grow from the walls of the auricle in *Blasia* and the slime cavity in *Anthoceros punctatus*, penetrating the cyanobacterial colony and facilitating nutrient exchange with the host plant (see: Adams 2002a; Adams and Duggan 2008). However, such ingrowths of the cells surrounding the *Nostoc* colonies are not found in many other hornworts, including *Leiosporoceros* (Villarreal and Renzaglia 2006). Once the hornwort slime cavity is colonised the middle lamella between internal cells separates to form an enlarged space,

allowing expansion of the colony; these changes do not occur in uninfected slime cavities (Renzaglia et al. 2000). Similarly, in *Leiosporoceros* the cavities containing the *Nostoc* are rarely seen in the absence of the cyanobacterium (Villarreal and Renzaglia 2006). The *Blasia* auricle also expands once infected by *Nostoc*, enabling the colony to grow (Renzaglia et al. 2000).

In cycads the entry of the cyanobacteria into the coralloid roots triggers significant morphological change, transforming them into typical infected coralloids (see: Costa and Lindblad 2002; Bergman et al. 2007a). Root diameter increases while elongation is reduced and the cells linking the inner and outer cortical layers become elongated, possibly to facilitate the exchange of nutrients between the partners (Costa and Lindblad 2002). These changes create a mucilage-filled space containing tightly-packed cyanobacterial filaments and traversed by elongated host cells which connect the original inner cortical layer and the newly-formed outer layer (Vessey et al. 2005).

In *Gunnera* the cells lining the gland channels divide in response to the presence of compatible cyanobacteria and it is thought that these are the cells that become infected (Bergman et al. 2007a). What induces these changes in *Gunnera* isn't known, although cyanobacteria do produce compounds involved in plant development (Liaimer and Bergman 2004) including the phytohormone auxin, indole-3-acetic acid (Sergeeva et al. 2002). Adaptations specifically for nutrient exchange aren't needed in *Gunnera* because the intracellular location of the cyanobionts ensures efficient transfer of nutrients. Similarly, in the *Geosiphon* symbiosis the location of the cyanobionts within the specialised bladder is sufficient to ensure good nutrient exchange. In lichens, it is the close association between the cyanobiont and thin-walled fungal hyphae, often without contact between fungal and cyanobacterial cell walls, that ensures efficient nutrient exchange (Rikkinen 2002). In a few cases, fungal haustoria penetrate the cyanobiont cell wall and enter the cells.

23.6.3 N_2 Fixation and Transfer of Fixed Nitrogen

In many cyanobacterial symbioses the role of the cyanobiont is to provide combined nitrogen for the host. This is particularly true of plant symbioses in which all known cyanobionts are capable of N_2 fixation (Kneip et al. 2007; Bergman et al. 2007a). By contrast, in the sponge symbioses the cyanobiont role is primarily provision of photosynthate. Nevertheless, although no heterocystous cyanobacteria have been reported as cyanobionts of sponges, there is evidence of N_2 fixation by sponge cyanobionts. Using PCR and RT-PCR, both the presence of and expression of *nifH*, encoding dinitrogenase reductase, one of the two nitrogenase proteins, has been demonstrated in two marine cyanosponges (Mohamed et al. 2008), implying that cyanobacteria may benefit the sponge by provision of fixed N (Taylor et al. 2007). In the ascidian-cyanobacteria symbiosis early reports of N_2 fixation by *Prochloron* have not been confirmed (Carpenter and Foster 2002; Yellowlees et al. 2008). Ammonium is the primary nitrogenous waste of the host ascidian and this may be used by *Prochloron* as a source of N (Kühl and Larkum 2002; Yellowlees et al. 2008).

In many of the plant symbioses, such as the cycads, *Gunnera* and *Azolla*, the gradient of heterocyst frequency, from low in the youngest tissue to high in the oldest tissue is paralleled by a gradient of low to high nitrogenase activity, although the highest rate of N_2 fixation is often at intermediate heterocyst frequencies (Meeks and Elhai 2002; Meeks 2003, 2009; Bergman et al. 2007a). Such a gradient of N_2 fixing capacity is particularly apparent in the stolon of *Gunnera magellanica*, in which the lowest rate is found in newly-infected tissue and the highest in the region of the stolon where the heterocyst frequency is 20%. A corresponding increase in expression of genes involved in heterocyst development (*hetR* and *ntcA*) and nitrogenase (*nifH*) is also apparent (Wang et al. 2004) although it isn't known if these are the results of direct control by the plant, or are a consequence of the conditions experienced by the cyanobiont *in planta*. As heterocyst frequency continues to increase to 60% further along the stolon, the N_2 fixation rate declines (Bergman 2002; Bergman et al. 2007a). This decline in rate may result from poor transfer of photosynthate into heterocysts, particularly within the groups of adjacent heterocysts found in the regions of highest heterocyst frequency. This is probably because the cell envelopes of heterocysts are impermeable to gases and solutes (Walsby 2007) and so photosynthate must pass to the internal heterocysts of an adjacent group via the outer heterocysts.

In the *Azolla* symbiosis a gradient of increasing nitrogenase activity coincides with the increase in heterocyst frequency in the younger leaves along the main axis of the plant (see: Meeks and Elhai 2002; Bergman et al. 2007a; Meeks 2009). Nitrogenase is oxygen-labile and is protected in part by the thickened cell walls of the heterocyst and is thereby immune to the oxygen concentrations in the *Azolla* leaf cavity (Lechno-Yossef and Nierzwicki-Bauer 2002; Meeks et al. 2002). Oxygen concentration is lower in the midsection (where nitrogenase activity is high) compared with the apex and the base of the plant. Respiration of the whole symbiosis system probably accounts for the lowering of the oxygen concentration and might also be a valuable source of ATP for the reduction of molecular nitrogen (Lechno-Yossef and Nierzwicki-Bauer 2002), supported by elevated levels of ATP synthase and ferredoxin $NADP^+$ reductase in the cyanobiont (Ekman et al. 2008).

Nitrogen fixation in the endosymbiotic cyanobiont (the so-called spheroid body) of the diatom *Rhopalodia gibba* is strictly light dependent (Kneip et al. 2007) yet the closest relative of the cyanobiont is the unicellular N_2 fixing cyanobacterium *Cyanothece* which protects nitrogenase from oxygen inactivation by temporal separation of N_2 fixation (at night) and photosynthesis (by day). Light-dependent N_2 fixation in the diatom cyanobiont is possible because the cyanobacterium has lost the capacity for photosynthesis (and hence oxygen production) and instead is supplied with fixed carbon by the diatom. The symbionts in dinoflagellate *Histioneis depressa* label positively with anti-nitrogenase antibodies, so may be capable of N_2 fixation (Foster et al. 2006a).

23.6.3.1 Release of N to the Host

In many cyanobacterial associations much of the N_2 fixed by the cyanobiont is released to the host, the proportion retained by the symbiont varying considerably (Table 23.1) from as much as 50% in *Azolla* to as little as 10–20% in lichens, *Gunnera* and hornworts (Meeks and Elhai 2002; Rai 2002; Meeks 2003, 2009; Bergman et al. 2007a). Ammonia is the form of N released from the cyanobionts in liverworts, hornworts, *Azolla* and lichens (Rai 2002; Bergman et al. 2007a), ammonia and some asparagine in *Gunnera* (Bergman 2002; Bergman et al. 2007a), whereas in cycads the form is thought to be glutamine and possibly citrulline (Table 23.1; Costa and Lindblad 2002; Vessey et al. 2005).

Ammonia release can often be at least partly explained by decreased activity of glutamine synthetase (GS) in the cyanobiont (although in the cycad symbiosis this seems not to be the case; see below); this enzyme is part of the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway, which is the primary route of ammonia assimilation in cyanobacteria (Muro-Pastor et al. 2005; Flores and Herrero 2005). The decrease in GS activity in most symbiotically-associated cyanobacteria is achieved by a reduction in either the activity of the enzyme or in the amount of protein produced, and this varies in different hosts (Table 23.1). In hornworts, and presumably also liverworts, there is a reduction in the activity of GS (Meeks and Elhai 2002; Meeks 2003, 2009). The mechanism of this reduction isn't known, but it is likely to involve post-translational modification of the enzyme because there is little difference in the level of GS protein between *Anthoceros*-associated *Noctoc* and free-living cyanobacteria (Table 23.1; Meeks and Elhai 2002; Meeks 2003, 2009).

By contrast, the 70% reduction in GS activity in the *Azolla* cyanobiont can be accounted for by the very low levels of GS protein (Table 23.1), which in the *A. caroliniana* cyanobiont are 5–40% of those in free-living *Nostoc* and *Anabaena* strains (Rai et al. 2000; Pabby et al. 2004a; Meeks 2009). In addition, low levels of *glnA* (encoding GS) transcripts (10% of the levels found in the free-living cyanobacteria) have been reported, leading to the suggestion that a host-derived factor(s) selectively represses *glnA* expression in the cyanobiont (most recently discussed by Meeks 2009). Immunogold electron microscopy has revealed that GS concentration in the heterocysts of the *Azolla* cyanobiont is decreased to that normally associated with vegetative cells (Rai et al. 2000; Meeks 2009). More recently Ekman et al. (2008) also reported a reduction in the amount of GS protein in the *A. caroliniana* cyanobiont compared with a free-living *Nostoc* species. In summary, repression and/or lack of stimulation of GS synthesis (and possibly also the inactivation or inhibition of the remaining enzyme) limits ammonium assimilation in symbiotically-associated heterocysts, consequently leading to the release of ammonium into the *Azolla* leaf cavity, presumably via the vegetative cells as the heterocyst cell envelope is impermeable to gases and solutes (Walsby 2007).

In lichen cyanobionts the release of ammonia is a consequence of a reduction in the activity of both GS and GOGAT, the former by up to 90% as a result of a reduction in GS synthesis and hence the amount of the enzyme (Table 23.1; Rai 2002; Rai and Bergman 2002; Adams et al. 2006). By contrast, in the *Gunnera* cyanobiont the amount of GS protein is unchanged in symbiosis, but its specific activity is reduced to 70% of that in the free-living state (Bergman et al. 2007a; Table 23.1). Cycad cyanobionts release organic N (possibly glutamine and citrulline) rather than ammonia and have GS and GOGAT activities similar to those in free-living cyanobacteria (Costa and Lindblad 2002; Lindblad and Costa 2002; see below and Table 23.1).

Despite releasing much of the nitrogen they fix, cyanobionts do not show the physiological signs of nitrogen starvation, because they display characteristics associated with N excess, such as the presence of carboxysomes, cyanophycin granules and phycobilisomes which, under nitrogen deprivation, would be degraded to provide amino acids for protein synthesis (Meeks and Elhai 2002). Phycobiliproteins are both accessory photopigments and serve as a nitrogen reserve to be used during N starvation. Cyanophycin is a specialised N reserve consisting of a co-polymer of arginine and aspartic acid. Both of these are found in the cyanobionts of many plants hosts. For example, in the *Azolla* cyanobiont the percentage of vegetative cells containing cyanophycin is low in the apex (45%) and at the base of the axis (60%) but higher in the mid-section of the plant (80–85%) where heterocyst frequency and N_2 fixation rate are also higher (Lechno-Yossef and Nierzwicki-Bauer 2002; Zheng et al. 2009a).

23.6.3.2 Host Uptake of N

After release from the cyanobiont, the ammonia is taken up the GS-GOGAT pathway of the host in the case of bryophytes and *Azolla*, or by glutamate dehydrogenase in the case of the fungus in cyanolichens (Rai 2002; Bergman et al. 2007a). Plants have two types of GS isoenzymes, GS1 found in

the cytosol and GS2 that localises in plastids/chloroplasts. Immunogold labelling using an anti-*Azolla* GS2 antibody has shown that the *Azolla* GS occurs not only in chloroplasts but also in the cytoplasm of hair cells (Uheda et al. 2004). Moreover GS synthesis in the hair cells is specifically stimulated by the presence of the ammonium fixed and released by the cyanobiont. By contrast, in cyanobiont-free plants labelling of GS in hair cells is very weak (Uheda et al. 2004; Uheda and Maejima 2009).

The fixed nitrogen must next be transported throughout the host tissues. The major forms of N moving from the *Azolla* leaf cavity to the stem apex are thought to be glutamate (and possibly a glutamate derivative), glutamine and ammonia (Rai et al. 2000), whereas in cycads it is glutamine and citrulline, or in some cycad groups glutamine and glutamic acid, that are thought to be transported from roots to stem *via* the xylem (Costa and Lindblad 2002: Bergman et al. 2007a). Alanine is thought to be the form in which N moves from the cephalodia to the main thallus in tripartite lichens (Rai 2002; Bergman et al. 2007a). In *Gunnera monoica* fixed nitrogen is transported via the phloem (rather than the xylem as in other angiosperm-bacteria symbioses) from mature regions of the plant to apical regions and the leaves (Bergman et al. 2007a).

23.6.4 CO_2 Assimilation and Transfer of Carbon

In many cyanobacteria symbiotically associated with a photosynthetic host the rate of light-dependent CO_2 fixation is greatly reduced from the free-living state and the cyanobiont grows photo- or chemo-heterotrophically using fixed carbon supplied by its host. The primary route for the fixation of CO_2 in cyanobacteria is the Calvin-Benson-Bassham cycle, and ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) is the primary carboxylating enzyme. A reduction in the specific activity of this enzyme, by an unknown mechanism, is responsible for the reduction of CO_2 fixation in some, but not all, plant symbioses (Table 23.2 and see below). The mechanism of inhibition may well vary in different symbioses.

Immediately following isolation from *Anthoceros* the *Nostoc* symbiont shows only 12% of the light-dependent CO_2 fixation of the free-living cyanobacterium (Table 23.2). This reduced activity seems to be the result of a post-translational modification of the Rubisco protein, as the amount of protein differs little between free-living or *Anthoceros*-associated *Nostoc* (Meeks and Elhai 2002; Meeks 2003). Because of its low CO_2 fixation capacity the *Nostoc* cyanobiont requires a supply of sugars from the host (Meeks and Elhai 2002; Meeks 2003).

The cyanobionts of cycads receive little if any light and are assumed to receive their carbon from the host, but the details are unknown (Costa and Lindblad 2002). Despite their heterotrophic nutrition and lack of CO_2 fixation, the cyanobionts retain Rubisco, which is active in extracts of the cyanobacteria freshly-isolated from the cycad (Table 23.2).

Table 23.2 Characteristics of light-dependent CO_2 fixation and ribulose bisphosphate carboxylase in symbiotically-associated cyanobacteria (Table compiled from Meeks (2009) and Adams (2000) and references therein)

Host	Light-dependent CO_2 fixation (%)	Rubisco	
		Protein (%)	Specific activity (%)
Cycads	0	ND	87–100
Gunnera	<2	100	100
Hornwort (*Anthoceros punctatus*)	12	100	12–15
Azolla	85–100	ND	ND
Lichens:			
Bipartite	ND	~100	ND
Tripartite	~8	~100	~8

Percentages indicate the value in the cyanobiont immediately after isolation from the host, compared with that in the free-living strain
ND not determined

In the *Gunnera-Nostoc* symbiosis it appears that rather than a total loss of the cyanobiont cellular photosynthetic machinery, there are modifications of key components (such as the D1 protein of the photosystem II complex) to render the pathway inactive (Black and Osborne 2004). This shift signals a change from a photoautotrophic to a heterotrophic metabolic state, perhaps triggered by the presence of fixed carbon from the plant, possibly in the form of glucose or fructose (Black et al. 2002; Black and Osborne 2004). Indeed, fructose and glucose support dark N_2 fixation in the free-living *Gunnera* isolate *Nostoc* PCC 9229 and after 4 weeks dark growth on fructose multiple adjacent heterocysts are found, together with high expression of *hetR*, reminiscent of the symbiotic growth state (Wouters et al. 2000). In addition, *Nostoc* PCC 9229 produces a putative cyclodextrin glucosyl transferase in the light and dark when fructose is supplied (Wouters et al. 2003). This enzyme is a member of the α-amylase family and typically catalyses the hydrolysis of, for example, α-D-glucose.

In *Azolla*, photosynthetic activity and CO_2 fixation by the cyanobiont are significantly reduced, contributing less than 5% of the photosynthetic oxygen evolution and CO_2 fixed in the association (Meeks 2009). Transcripts for Rubisco are also some 5–7-fold lower in the cyanobiont compared with free-living cultures (see: Adams 2000; Rai et al. 2000; Meeks 2009). Similarly, proteomic analysis has revealed lower levels of Rubisco in the *Azolla* cyanobiont than in cultured *Nostoc* PCC 73102 (Ekman et al. 2008). Surprisingly, immediately after isolation from the plant, the primary cyanobiont has approximately 85% of the photosynthetic rate of free-living cyanobacteria (Table 23.2); why this should be is not known. Energy requirements of the cyanobiont are believed

to be met in part by carbohydrates, mainly sucrose or possibly fructose, supplied by the host (Ekman et al. 2006). Correspondingly, Ekman et al. (2006) revealed an up-regulation in key enzymes potentially involved in the assimilation of host-derived carbon sources, including a likely hexose transporter that was four times more abundant in the *Azolla* cyanobiont than its homologue in the free-living *Nostoc* strain. The oxidative pentose phosphate pathway (OPP) is the major route of carbon catabolism in cyanobacteria and is a source of reductant to both nitrogenase and oxidative respiration. Elevated levels of the OPP enzyme 6-phosphogluconate dehydrogenase in the *Azolla* cyanobiont may be a reflection of the higher demands for reductant during symbiotic growth (Ekman et al. 2008).

Cyanobionts of bipartite lichens transfer 70–80% of the carbon they fix, in the form of glucose, to the fungal host, whereas in tripartite lichens the cyanobiont transfers little, if any, fixed carbon to the mycobiont (Palmqvist 2000, 2002; Adams et al. 2006; Bergman et al. 2007a). The transferred glucose is rapidly converted to mannitol, which can only be used by the mycobiont. In tripartite lichens cyanobionts in cephalodia located within the cortex or underneath the thallus may receive so little light that they are not photosynthetically active and so must receive carbon from the primary photobiont (Palmqvist 2002; Bergman et al. 2007a). The rate of CO_2 diffusion is 10,000 times lower in water than in air, and so the hydration state of the thallus greatly affects photosynthesis (Palmqvist 2002). This is because a damp thallus still has air spaces through which CO_2 can diffuse rapidly, whereas these spaces are filled with water in a wet thallus and swelling of the fungal hyphae can also block gaseous pores. Cyanolichens can be at an advantage in wet conditions because they have a CO_2-concentrating mechanism that can at least partly compensate for the reduced CO_2 diffusion (Palmqvist 2002). However, even cyanolichens vary in their ability to photosynthesise under very wet conditions (Lange et al. 2004).

In the *Geosiphon* symbiosis the function of the *Nostoc* is primarily photosynthesis and accordingly its photosynthetic activity is greater than in the free-living state (Kluge 2002; Adams et al. 2006). Carbon is transferred from *Nostoc* to the fungal host possibly in the form of sucrose, although it has been suggested that degradation of *Nostoc* extracellular polysaccharides may release hexoses that could be transported into the fungus by the hexose transporter GpMST1 found in *Geosiphon pyriformis* (Shüssler et al. 2006)

In the remaining cyanobacterial symbioses the details of symbiont CO_2 fixation and transfer of carbon to the host are poorly understood. Cells of the *Prochloron* cyanobiont of ascidians contain ribulose bisphosphate carboxylase in carboxysomes and CO_2 uptake is aided by carbonic anhydrase activity (Griffiths 2006; Yellowlees et al. 2008). Carbon photosynthetically fixed by *Prochloron* can be transferred to the host and, in *Didemnum molle* and *Lissoclinum patella*, may provide between 12% and 56% of its reduced carbon requirement (Griffiths 2006; Yellowlees et al. 2008). In the marine diatom *Rhizosolenia* the cyanobiont, *Richelia intracellularis*, provides its host with both N and C (Adams 2000). By contrast the unicellular cyanobionts of the dinoflagellates *Ornithocerus magnificus* and *Ornithocerus steinii*, appear to provide only fixed carbon for their non-photosynthetic host. In the case of sponge-cyanobacteria symbioses carbon transferred from the cyanobiont to the sponge, possibly as glycerol and organic phosphate, can satisfy up to 50% of the host's energy requirement and 80% of its carbon budget (Erwin and Thacker 2007; Usher 2008).

23.7 Artificial Cyanobacteria-Plant Symbioses

There have been numerous attempts to construct novel associations between plants and cyanobacteria, the goal being the replacement of at least a proportion of current artificial nitrogenous fertiliser used for crop plants, with the resultant commercial and environmental benefits. This work has so far involved the introduction of cyanobacteria into higher plant protoplasts (see: Rai et al. 2000; Adams 2000; Gusev et al. 2002; Bergman et al. 2007a), or the co-culture of cyanobacteria with plant tissue cell cultures, plant regenerates and cuttings from a variety of plants (Gantar 2000b; Gorelova 2001, 2006; Lobakova et al. 2001a, b; Gorelova and Korzhenevskaya 2002; Gorelova and Kleimenov 2003; Gorelova and Baulina 2009; see also Rai et al. 2000 and Gusev et al. 2002 for a discussion of the earlier literature). Some *Nostoc* strains have been shown to be attracted to exudates from plants that do not normally serve as hosts (Nilsson et al. 2006) and to colonise the surface of the roots of rice (Nilsson et al. 2002; 2005) and wheat (Karthikeyan et al. 2007, 2009; Sood et al. 2011). Mechanical damage of wheat seedling roots can result in growth of cyanobacteria within the root tissues (Gantar 2000a) and in laboratory tests the colonization of wheat roots by some heterocystous cyanobacteria can lead to enhancement of plant N content and root growth (for a discussion of this see: Rai et al. 2000; Adams 2000; Gusev et al. 2002; Bergman et al. 2007a).

23.8 Concluding Remarks

This chapter has discussed the many known cyanobacterial symbioses, but where might there be new ones waiting to be discovered? Novel cyanobacteria-plant associations are perhaps most likely to occur where the conditions for the survival of hormogonia are optimum. For the infection of plant roots this may be in almost any soil, but for the infection of stems or leaves this is likely to require a good level of

moisture, such as that found in temperate and tropical rain forests and in wet boreal forests where mosses thrive. These moist environments are also ideal for the survival of epiphytic cyanobacteria which may supply combined nitrogen to the plant itself or to the local ecosystem, as is the case for the cyanobacteria-moss associations. Perhaps in such wet and humid environments might be found a modern-day higher plant equivalent of the extinct *Aglaophyton major-Archaeothrix* symbiosis in which hormogonia infected the leaves via stomatal pores in a manner similar to the extant hornwort *Leiosporoceros dussii*. The oceans are also likely to be a major source of undiscovered cyanobacterial symbioses, an interesting potential example of which is the marine, N_2 fixing unicellular cyanobacterium UCYN-A (Zehr et al. 2008; Tripp et al. 2010) which is evolutionarily related to the spheroid bodies of *Rhopalodia gibba* (Bothe et al. 2010). This cyanobacterium lacks the photosystem II complex and the biosynthetic pathways for several amino acids and purines, implying that it is reliant on a symbiotic partner, although such a partner has yet to be identified.

Another question to consider is the potential for the future agricultural use of cyanobacteria-plant symbioses. Only *Azolla* has been used as a green manure in agriculture. However, N only becomes available to the rice plants when *Azolla* decays. A more efficient delivery of combined nitrogen to a crop would require the growth of cyanobacteria on the surface of, or within the plant. Such symbioses do not exist, so what is the likelihood of creating them artificially? As N_2 fixing symbionts, cyanobacteria such as *Nostoc* spp. have the great advantage that they possess, in the heterocyst, a unique system for the protection of nitrogenase from oxygen inactivation. They also show a catholic taste in hosts and have a highly developed infective agent, the hormogonium. *Nostoc* spp. would therefore make ideal N_2 fixing symbionts for the creation of novel symbioses with crop plants. To avoid the need to re-establish the partnership at each generation these symbioses would ideally be self-perpetuating. Yet, despite the (presumed) long evolutionary history of cyanobacteria-plant symbioses, *Azolla* is the only example in which the cyanobiont is passed from one generation to the next. It seems likely therefore, that the chance of creating novel, self-perpetuating cyanobacterial endosymbioses with crop plants, is not high (Bergman et al. 2007a). In addition, because the cyanobiont in all cyanobacteria-plant symbioses occupies existing structures (although these can undergo modification following infection) plants lacking suitable structures will not provide ready hosts. A further problem is that modern cereal crop varieties have been bred for rapid growth, requiring high rates of N input for short periods, and cyanobacteria are likely to provide at best only a portion of the N required (Bergman et al. 2007a). The introduction of cyanobacteria to a slower-growing crop plant may therefore be most effective at fulfilling the N requirements of the plant.

In the laboratory, *Nostoc* spp. have been shown to colonise the roots of wheat seedlings and enhance root and plant growth. It isn't known if such interactions occur in the field, but if they do, then a relatively simple strategy would be to enhance these existing interactions by producing plant varieties that release larger amounts of the chemical signals that induce and attract hormogonia, thereby stimulating colonization of roots to the benefit of the plant. What is lacking at present is knowledge of the chemical identity of these plant signals. This approach would have the benefit of not requiring the introduction of mutant cyanobacteria into the field where they would be unlikely to compete with natural populations.

Another stumbling block to the creation of artificial cyanobacteria-plant symbioses is our poor understanding of the involvement of the plant partner in existing symbioses. Most research effort has focussed on the cyanobiont, with relatively little attention given to the plant, perhaps because such work is more technically difficult. We need a much clearer understanding of the signals exchanged by the partners, because the ability to produce and respond to these signals will be vital to the chances of any cyanobiont forming a stable symbiosis with a potential plant host. Indeed, the scarcity of plant cyanobionts from some heterocystous- and hormogonia-producing genera may be a result of their inability to respond appropriately to plant signals.

Finally, what is the environmental impact of cyanobacterial symbioses? It is becoming clear that many, by virtue of their capacity for N_2 fixation, are of major environmental significance. Many of the known marine cyanobacterial symbioses, such as those with diatoms and dinoflagellates, are poorly understood, yet their abundance and N_2 fixing capacity can have a major impact on N availability in the open oceans. On a smaller scale, benthic N_2 fixing cyanobacterial symbioses with sponges and sea grasses can supply significant amounts of N to their local ecosystems. The impact of N_2 fixing cyanobacterial symbioses is of course greatest where alternative N inputs are least; so, cyanolichens and cyanobacteria-moss associations can have major impacts in many regions of the northern hemisphere where inputs of combined nitrogen from N_2-fixing plants and atmospheric deposition are low. Of course future climate change may have significant impacts on these symbioses and the ecosystems that their N_2 fixation supports.

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