

## Review

# On the origin of plants and relations to contemporary cyanobacterial-plant symbioses

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**Abstract** Cyanobacteria are highly versatile organisms in spite of their small size; they represent the smallest photosynthetic units on Earth, and gave rise to algae and plants. Cyanobacteria are globally widespread in terrestrial and aquatic environments, including the large oceans. In recent years they have attracted attention because of their high evolutionary importance, their developmental complexity, their unexpected pivotal ecological significance in global biogeochemical cycles and their production of a large number of secondary metabolites, many with great biotechnological potential. In this overview, focus is on the role of cyanobacteria in the origin and evolution of algae and plants, and on mechanisms in contemporary cyanobacterial-plant symbioses (*Gunnera* and *Azolla*) that may represent ‘blue-prints’ of those that some 2.1 billion years ago became the first chloroplasts.

**Key words:** Cyanobacteria, plant evolution, symbiosis, secondary metabolites, toxins

## *Cyanobacteria: the smallest photosynthetic unit*

Cyanobacteria originated about 3 By ago (Schopf 2000) and were the first to introduce oxygenic photosynthesis on Earth. In doing so, they acquired an important mechanism to sequester the key nutrient carbon, from atmospheric carbon dioxide, solely using light energy captured by pigments, such as chlorophyll *a* and the chromatically complementary phycobiliproteins (phycoerythrin and phycocyanin). Their evolutionary and ecological success was thereby given. A side effect of this reaction was the splitting of water into oxygen, which led to a change from the reducing atmosphere prevailing at that time, into the oxidative atmosphere we experience today (~20% O<sub>2</sub>): a truly dramatic shift for all living organisms.

A broad morphological diversity is seen in cyanobacteria, ranging from unicellular individuals to multicellular representatives with branched or unbranched chains of cells (Rippka et al. 1979). They are classified into Sections I and II, with unicellular cyanobacteria differing in their division patterns, Section III with filamentous cyanobacteria composed of vegetative cells only; and into Section IV and V containing the most developmentally advanced cyanobacteria, the latter section with filament branching. All representatives of Section IV and V are capable of transforming their photosynthetic vegetative cells into other cell types with altered physiological roles as a

response to changes in external conditions (Adams and Duggan 1999). These two Sections in fact hold the geno- and phenotypically most complex prokaryotes known. The development of additional cell types provides means to sequester incompatible physiological processes into different cellular compartments (see below). This developmental capacity may also represent a ‘primitive’ first stage of what later evolved into more elaborate and highly specialized organs, as we today know from eukaryotic organisms. Cyanobacteria may therefore represent excellent ‘model systems’ for understanding basic mechanisms involved in cell differentiation, organ development and physiological specialization in more complex organisms.

Besides carrying out oxygenic photosynthesis in vegetative cells, the lack of combined nitrogen in some cyanobacteria induce the differentiation of a cell type solely devoted to uptake of atmospheric nitrogen (nitrogen fixation). There are two types of nitrogen-fixing cells: heterocysts, a character of Section IV and V cyanobacteria (Adams and Duggan 1999) and diazocytes, a novel cell type detected in the Section III marine cyanobacterium *Trichodesmium* (Berman-Frank et al. 2001; El-Shehawy et al. 2003). Some cyanobacteria in Section IV and V may also change their entire set of vegetative filaments into small-celled filaments, termed hormogonia, possessing gliding motility, chemotactic movement and floating capacity. To survive extreme

stress, the vegetative cells may also develop into resilient resting akinetes or spores (Adams and Duggan 1999).

Due to these differentiation capacities, some cyanobacteria are able to survive seasonal variations, to thrive in many diverse terrestrial and aquatic ecosystems, and to grow in extremely nutrient poor environments such as that of the Antarctic, terrestrial hot springs, dry and hot deserts, the nutrient deficient surface waters of oceans; and indeed, inside plants and even inside plant cells (Whitton and Potts 2000).

### The origin and evolution of plant chloroplasts

A most prominent capacity of cyanobacteria is their ability to interact and establish long-lived symbiotic associations with a few but diverse representatives among fungi and within the plant kingdom (Adams et al. 2006; Bergman et al. 2007, 2008). In the history of earth, a symbiotic interaction of greatest evolutionary significance is without doubt the merger of some cyanobacteria with some non-pigmented eukaryotes about 2.1 billion years ago (Raven 2002). This merger gave rise to algae and subsequently, about 500 to 450 million years ago, to lichens and all land plants (see Usher et al. 2007). This is a second most fundamental step in the creation of our biosphere involving cyanobacteria (the first being the oxygenation of the atmosphere). The engulfed cyanobacterium brought the photosynthetic process into a new context, the *Eukarya* domain, and once in there, cyanobacteria eventually evolved into chloroplasts. Still today, cyanobacteria and plants share the same fundamental basic characteristics in their photosynthesis. This gigantic evolutionary step of cyanobacteria being incorporated in eukaryotes, giving rise to green plants, is considered as a monophyletic event. The organisms that originally participated in this merger, and mechanisms that operated during the process are, not unexpectedly, still unknown. The introduction of molecular techniques and novel exciting model systems is however starting to unravel some aspects.

It is apparent that these original pro- and eukaryotic partners efficiently co-evolved into becoming a most successful new entity (algae/plants) through endosymbiosis. From being a free-living cyanobacterium chloroplasts of green plants have a dramatically changed life-style, although the fact that they still retain their own cpDNA (chloroplastic DNA) reveals their prokaryotic origin. The genomes of contemporary chloroplasts are in the size of 150–200 kbp (Raubeson et al. 2005), hence severely diminished from the ~1–10 Mbp genome size of the potential cyanobacterial progenitor. Through the course of evolution of chloroplasts, redundant cyanobacterial genes were lost and/or transferred to the

plant nucleus (Martin et al. 2002; Deusch et al. 2008). By now, more than fifty chloroplast genomes have been sequenced from land plants, the majority of which are from the flowering plants, including some from globally agriculturally important crop plants such as rice (Raubeson and Jansen 2005; Daniell et al. 2006). A high conservation exists among all chloroplast genomes sequenced so far. Besides retaining photosynthetic capacities as well as the transcription and translation machinery (rRNAs and a few tRNAs), most chloroplasts maintain some metabolic activities, for instance the synthesis of certain amino acids, lipids, pigments and starch. The availability of whole chloroplast genome sequences has stimulated evolutionary studies and advanced genetic engineering related to plant breeding. Transformations of the small chloroplast genomes may offer advantages over nuclear transformation to for instance improve crop plant performances (Daniell et al. 2005).

One scientific challenge is to robustly link the chloroplasts to contemporary cyanobacterium(a) in order to reveal the closest extant relative to the original cyanobacterium, the progenitor of chloroplasts. The ‘unicellular’ character of chloroplasts has led to a previous assumption that some unicellular cyanobacterium (from Section I or II) most likely acted as the ancestral cyanobacterium entering the ‘plant-to-be’. Indeed, comparative *rpoC1* gene (encoding a subunit of DNA-dependent RNA polymerase) and 16s rRNA analyses implied that the so called ‘cyanelles’, cyanobacterium-like plastids of some protists, which still retain a rudimentary cyanobacterial cell wall, were the closest relatives to chloroplasts, when compared to representatives from prochlorophytes (cyanobacteria with chlorophyll *a* and *b*) and some free-living cyanobacteria (Palenik and Haselkorn 1992; Urbach et al. 1992). However, the most recent evolutionary analyses suggest that this may not be the case. Novel data from genome comparisons, embracing a markedly larger number of pro- and eukaryotic organisms with sequenced genomes, imply that a cyanobacterium with more complex morphology and nitrogen-fixing heterocysts, acted as the chloroplast ancestor (Martin et al. 2002; Deusch et al. 2008). These findings open new perspectives on the origin of the ancestor of chloroplasts in green plants.

### Cyanobacterial-plant symbioses

The group of contemporary cyanobacteria that today readily interacts with eukaryote hosts to form long-lived symbiotic interactions most often belong to Section IV cyanobacteria (Rasmussen and Nilsson 2002; Adams et al. 2006). This strongly supports the above mentioned genetic findings using the large data set of sequenced

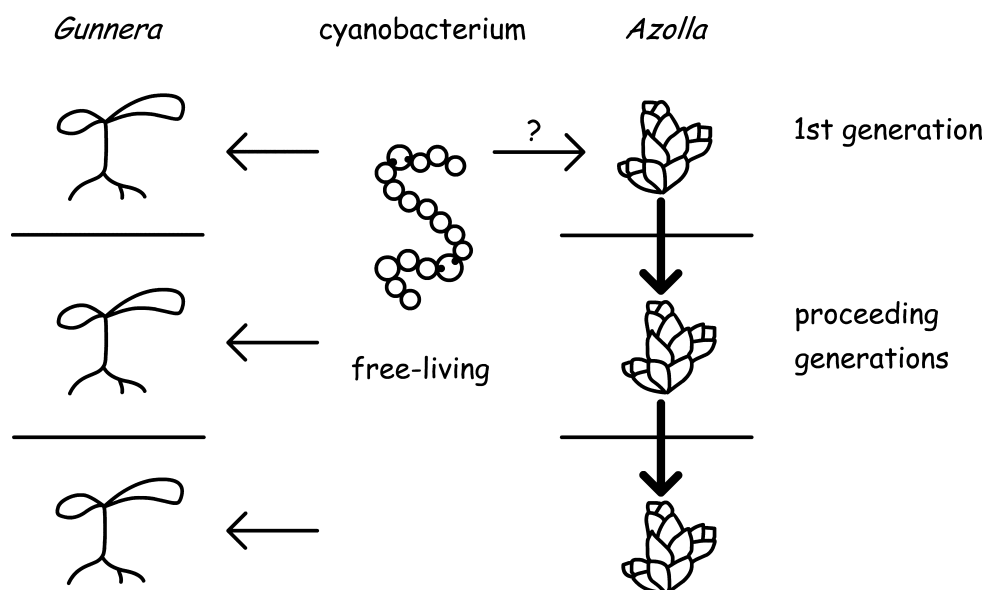


Figure 1. *Gunnera* stem cells need to get colonized by cyanobacteria for each new plant generation (thin arrow), while the *Azolla* symbiosis is perpetual (thick arrow), with vertical transmission of the cyanobacterium between plant generations. A higher degree of co-evolution between the cyanobacterium and the plant is therefore expected in the *Azolla* symbiosis, since a stable symbiotic environment inside the *Azolla* leaf cavity has been provided during the (potentially) millions of years the symbiosis has existed. It is uncertain when the first cyanobacterial symbiosis was established with *Azolla*, as indicated by a question mark.

genomes from plants, algae and prokaryotes (Martin *et al.* 2002; Deusch *et al.* 2008). The nitrogen-fixing cyanobacterial genus *Nostoc* occurs in the majority of present-day plant symbioses. The model cyanobacterium, *Nostoc* PCC 73102 (equivalent to *Nostoc* ATCC 29133 and *N. punctiforme*), is an organism with well-documented symbiotic competence that readily colonizes symbiotically competent plants (Meeks *et al.* 2001; Bergman 2002; Bergman *et al.* 2007). We have isolated a large number of other *Nostoc* species/strains from plant symbioses which show similar symbiotic characteristics. The high symbiotic competence shown by *Nostoc* species may therefore have persisted through the 2.1 billions of years of evolution since the first 'encounter'.

The engulfment of this second cyanobacterium is apparently not to acquire additional CO<sub>2</sub>-fixing chloroplasts (except in a few lichens, see Bergman *et al.* 2007), but to acquire nitrogen-fixing "nitroplasts", providing its host with the second most important macro-nutrient, nitrogen (Bergman *et al.* 2007, 2008). We have therefore set up a research programme in which selected contemporary cyanobacterial-plant symbioses are used as model systems to get a deeper knowledge about inter-kingdom interactions and the evolution of symbiosis. In lack of any direct evidence in the fossil record from the ancient endosymbiotic event that led to green plants, the examination of present day symbiotic actors and their interactions may give clues about the evolution of chloroplasts, and shed light on their genetic origin and on mechanisms involved in the establishment of the green plants. Although cyanobacteria establish

symbioses with a range of fungi (non- and lichenized) and plants (bryophytes, pteridophytes, gymnosperms and angiosperms), we have in our research selected two symbiotic systems of particular interest in this context, the angiosperm *Gunnera* and the fern *Azolla* (Bergman 2002; Lechno-Yossef and Nierzwicki-Bauer 2002; Bergman *et al.* 2008). These symbioses hold traits potentially related to the creation and evolution of the plant kingdom. The former is unique as it represents the only plant endosymbiosis, that is, the cyanobacterium enters through the cells walls of the host plant as motile hormogonia, which inside the plant cells develop into vegetative filaments with a high heterocyst frequency. The cyanobacterial-*Azolla* symbiosis, on the other hand, is unique as it is the only perpetually maintained cyanobacterial-plant symbiosis. A mechanism has evolved that allow transmission of a small cyanobacterial 'inoculum' from one to the next plant generation. This is unlike all other cyanobacterial-plant symbioses, including the *Gunnera* symbiosis, in which re-infection of each new plant generation is required. These two cyanobacterial-plant symbioses may represent the most evolutionary advanced, and the only endocytotic and the first obligate inter-dependent type of plant symbiosis, respectively.

In all plant symbioses the fixed nitrogen is released and delivered to cover nitrogen needs of the host plants (Bergman *et al.* 2008). In this way, host plants get access to two macro-nutrients via domestication of basically the same cyanobacterium by promoting two different physiological processes. This requires a fine-tuned



co-evolution between organisms representing different domains of life.

**The cyanobacterial-angiosperm symbiosis, *Gunnera***

The only angiosperm that serves as a host for a nitrogen-

fixing cyanobacterium is the genus *Gunnera* (Bergman 2002). These plants develop specialized stem glands that serve as entry portals for symbiotically competent cyanobacteria living in the surrounding soil. These portals are however selective, allowing only compatible cyanobacteria to reach the intracellular stage, although

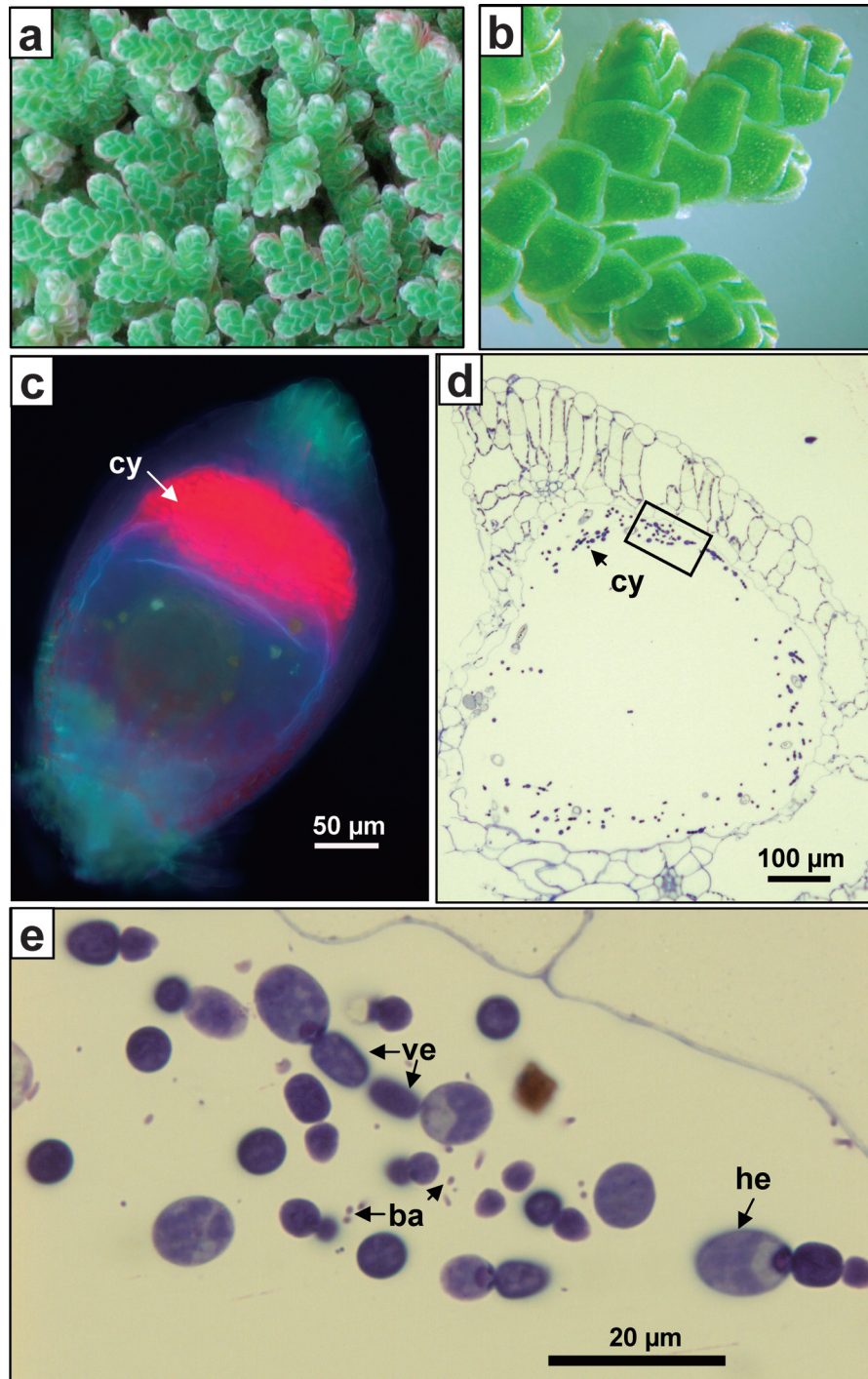


Figure 2. The cyanobacterial-*Azolla* symbiosis. a) The free-floating *A. filiculoides*; b) an enlargement showing the dorsal leaves in which the cyanobacterium occupies a cavity; c) fluorescence micrograph of a megasporocarp infected by cyanobacteria fluorescing red (cy); d) LM-micrograph of a cross sectioned *A. filiculoides* leaf cavity showing cyanobacterial filaments (cy) close to the wall of the cavity; e) enlargement of the boxed part in (d), showing cyanobacterial filaments composed of vegetative cells (ve) and nitrogen-fixing heterocysts (he). Note also some small-sized heterotrophic bacteria (ba).

bacteria and fungi are present at the gland surface. Mechanisms involved in this discrimination and the subsequent phagocytosis of *Nostoc* are largely unknown. A similar mechanism may have operated in the creation of plants and led to its monophyletic character. Cyanobacterial cell surface components may play a pivotal role for such early contact (motility, chemotaxis, attachment, recognition, entry, etc) and for other early stages during the establishment of cyanobacterial-plant symbioses in general. Cyanobacteria that gain entry into plants are likely to have developed mechanisms as sophisticated as those used by pathogenic bacteria or pollen, such as proteins that function as receptors for plant cell surface components. Or they may “hide” behind plant-like cell surface components to avoid being recognized as ‘non-self’ and evoking plant defence responses.

Cyanobacteria that naturally associate with *Gunnera* promptly adapt to the plant conditions offered inside the plant, but regain their free-living competence equally fast on isolation (Bergman 2002; Bergman *et al.* 2007). Hence, the adaptive responses in the cyanobacterium represent only a transient stage, which however lead to diverse phenotypic changes culminating in those that best fit the plant. Such changes include the differentiation of vegetative cells into motile hormogonia, which is known to be induced by plant compounds. The hormogonia are used as vehicles to reach the sessile plants. Using comparative proteomics, a few proteins/genes have been identified that are involved in hormogonium development, many of which are surface related or secreted (Klint *et al.* 2006), or related to the central carbon metabolism. (Ran L, Klint J, Duggan P, Liaimer A and Bergman B, unpublished data).

Prior to the entry of the hormogonia into plant cells in the interior of the *Gunnera* stem glands, these plant cells are induced to divide by the presence of compatible cyanobacteria (Bergman *et al.* 2008). This process may be stimulated by the plant hormone auxin (possibly in combination with other hormones) produced by the entering cyanobacteria (Sergeeva *et al.* 2002). Colonization of the host plant cells is followed by the differentiation of nitrogen-fixing heterocysts at frequencies that are several-fold higher than under any free-living conditions (Bergman *et al.* 2007). As differentiation into hormogonia leads to a dramatically increased cell division rate, the expression of cell division genes and corresponding proteins (e.g. *ftsA/ftsZ*) have been examined and found to be stimulated (Ran L, Klint J, Duggan P, Liaimer A and Bergman B, unpublished data). In contrast, cell division of the cyanobacterium is dramatically slowed down and the cells enlarged when hormogonia enter plants, indicative of a blocked cell division and potentially of an endoreduplication of their DNA. Our studies collectively

show that prerequisites for the establishment of an endosymbiosis are a chain of adaptations and molecular mechanisms, targeting basic cellular processes in the cyanobacterium such as cell division and cell differentiation as well as physiological performances (Wang *et al.* 2004; Ekman *et al.* 2006; Ran *et al.* 2007; Bergman *et al.* 2007).

### ***The cyanobacterial-fern symbiosis, Azolla***

The second cyanobacterial-plant symbiosis to be discussed here is the symbiosis between cyanobacteria and the small free-float water-fern. *Azolla*, which shows strikingly unique symbiotic characters which are of relevance for our understanding of the evolution of symbiosis and plant. In contrast to the cyanobacterial-*Gunnera* symbiosis, cyanobacteria in *Azolla* reside extracellularly in dorsal ‘cavities’ of the *Azolla* leaves (Peters and Meeks 1989; Lechno-Yossef and Nierzwicki-Bauer 2002). The *Azolla* leaves are in nature constitutively colonized by filamentous nitrogen-fixing cyanobacteria, the classification of which is still uncertain, but the cyanobionts belong to either the genus *Nostoc* or perhaps *Anabaena* (Lechno-Yossef and Nierzwicki-Bauer 2002; Papaefthimiou *et al.* 2008).

A key mechanism in the evolution of photosynthetic chloroplast must have been making the cyanobiont unable to escape and perhaps more importantly, to make it persist between plant generations to ascertain the access to a renewable carbon source, formed using solar energy only. Interestingly, the *Azolla* symbiosis is the only plant symbiosis in which a strategy that secures a transfer of the cyanobacterium to the next plant generation has developed. The sexual reproduction of the *Azolla* plant is initiated by the development of mega- and micro-sporocarps, the male and female reproductive organs of *Azolla* (Zheng *et al.* 1988). These form in pairs at the underside of the *Azolla* plants. At a very early stage of the mega-sporocarp development, motile hormogonium filaments originating from nearby *Azolla* leaf cavities, by some unknown mechanism, enter and get packed into small ‘chambers’ inside the megaspore and the indusium chamber. Hence, a vertical transfer between plant generations. The fact that it has proved difficult to grow *Azolla* cyanobiont after isolation under any laboratory conditions suggests that the cyanobacterium has lost its free-living capability. It may have lost or silenced parts of its genome during the co-evolution of the symbiotic partners. These two features of the *Azolla* cyanobiont make knowledge about its genome sequence of tremendous interest: the *Azolla* symbiosis may be on its way to evolve into the first  $N_2$ -fixing plant, with a new kind of ‘organelle’. Sequencing of the cyanobiont genome is now underway (DOE\_JGI, USA). Meanwhile, attempts have been made to disclose molecular and morphological hallmarks and adaptations



of the *Azolla* cyanobionts using a proteomic approach (Ekman et al. 2006). Although the genome of the *Azolla* symbiont is not yet available, numerous up- or down-regulated proteins were identified making use of the genome sequences of closely related cyanobacteria *Nostoc* PCC 73102 (Meeks et al. 2001) and *Anabaena* PCC 7120 (Kaneko et al. 2001), which are publicly available. Proteins affected were related to energy production, nitrogen and carbon metabolism, stress-related functions and photosynthesis (Ekman et al.

2008).

Collectively, the data obtained so far using these symbiotic systems suggest that plants are capable of inducing several cellular and molecular adaptations in prokaryotes and to the extent that their symbiotic phenotypes are not seen when the cyanobacteria are free-living. Similar adaptations must have been operative in the cyanobacterium that became the first endosymbiotic chloroplast. But instead of stimulating processes in the cyanobacterium related to the nitrogen metabolism, as is

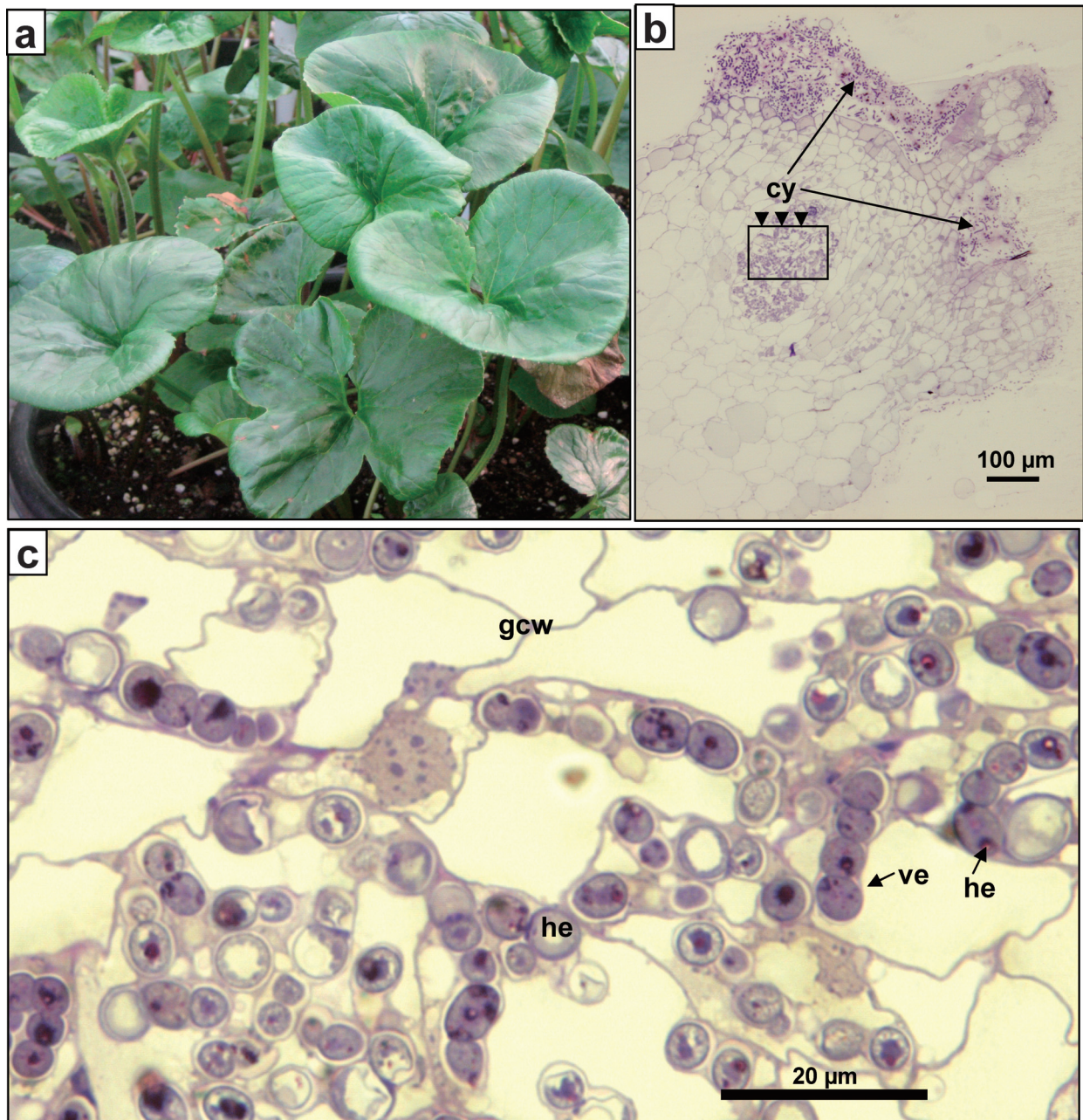


Figure 3. The cyanobacterial-*Gunnera* symbiosis. a) A *G. perpersa* plant; b) LM-micrograph of a cross sectioned infected stem gland of *G. manicata*. Cyanobacteria are seen both in the mucilage covering the outer surface of the gland (arrows) in the right hand part of the figure; and after penetrating the gland and occupying *Gunnera* cells located deeper in the gland (arrow heads); c) an enlarged micrograph of the boxed structure in (b), illustrating the intracellular localization of the cyanobacterial filaments interiorly of the *Gunnera* cell walls (gcw), the former with a high frequency of heterocysts (he).

the case in the *Gunnera* and the *Azolla* symbioses, the chloroplast progenitor was induced by its host to pursue photosynthesis, and perhaps to inhibit the differentiation of heterocysts. It is also clear that whole cyanobacterial filaments belonging to *Nostocales* can enter and function inside plant cells, albeit still outside the plant plasmalemma (the *Gunnera* symbiosis); and that one host plant has succeeded in developing a mechanism to transfer filamentous cyanobacteria between generations (the *Azolla* symbiosis). Further detailed analyses of these cyanobacterial symbioses are needed to gain additional insights into the origin of plant symbioses and of plants.

## Biotechnological aspects

Besides disclosing novel facts about symbiosis, inter-kingdom-interactions, and about evolution of cyanobacteria and plants, understanding cyanobacterial-plant symbioses is also pivotal for understanding plant biology and how to preserve, administer and make use of biological resources/diversity, for instance, alleviating our dependence on industrially produced nitrogen compounds, a production process consuming large amounts of fossil fuels. This applies to the interactions in both the endosymbiotic *Gunnera* and perpetual *Azolla* symbiosis. The cyanobiont-*Azolla* symbiosis is a globally wide-spread organism of great significance to agriculture as it has been used during centuries as a green manure in rice cultivation, and as fodder for domesticated animals (van Hove and Lejeune 2002). It also represents a uniquely fast-growing (a doubling in biomass every 3–4 days), environmentally friendly and self-renewable source of energy and key nutrients (C and N). It also has great potential for biotechnological uses, such as enhanced biomass production and bio-energy conversions (e.g. into bio-fuels) as its cyanobiont produces hydrogen gas, capabilities that potentially could be enhanced.

Another biotechnologically attractive goal is to use the symbiotic knowledge in attempts to create novel 'artificial' symbioses between plants of agricultural and economical interest and nitrogen-fixing cyanobacteria, in order to relieve our dependence on nitrogen fertilizers for food production. Few, but some promising, attempts have been made using symbiotically competent cyanobacteria to test their interactions with plants such as wheat and rice (Gantar *et al.* 1995; Watts *et al.* 1999; Nilsson *et al.* 2002), and cyanobacterial genes have recently been introduced into transgenic plants to enhance plant performance (Uchida *et al.* 2008).

Besides being efficient nitrogen-fixers, particularly so in symbiosis with plants, cyanobacteria have recently attracted attention as a source of new classes of diverse secondary metabolites and natural products (Ehrenreich *et al.* 2005; Devillers *et al.* 2007; Tan 2007). These

include highly cytotoxic and excitotoxic compounds such as  $\beta$ -N-methylamino-L-alanine (BMAA) produced by the majority of cyanobacteria including *Azolla* and *Gunnera* symbionts (Cox *et al.* 2005; see also Jansson *et al.* this issue). Their role as sources of vitamins and proteins is well recognized and cyanobacteria have for long been used and sold as health food and are used as fodder throughout the world. The role of cyanobacteria as potential antiviral, anti-tumour, antibacterial, anti-HIV agents has also been noted. A majority of these molecules are nitrogen-containing compounds, biosynthesized by large multimodular non-ribosomal polypeptide enzymatic systems. Taken together all these findings underscore the variety of potential novel biotechnologically important compounds to be exploited in free-living and symbiotic cyanobacteria.

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