Protein Expression Profiles in an Endosymbiotic Cyanobacterium Revealed by a Proteomic Approach

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Molecular mechanisms behind adaptations in the cyanobacterium (*Nostoc* sp.) to a life in endosymbiosis with plants are still not clarified, nor are the interactions between the partners. To get further insights, the proteome of a *Nostoc* strain, freshly isolated from the symbiotic gland tissue of the angiosperm *Gunnera manicata* Linden, was analyzed and compared with the proteome of the same strain when free-living. Extracted proteins were separated by two-dimensional gel electrophoresis and were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry combined with tandem mass spectrometry. Even when the higher percentage of differentiated cells (heterocysts) in symbiosis was compensated for, the majority of the proteins detected in the symbiotic cyanobacteria were present in the free-living counterpart, indicating that most cellular processes were common for both stages. However, differential expression profiling revealed a significant number of proteins to be down-regulated or missing in the symbiotic stage, while others were more abundant or only expressed in symbiosis. The differential protein expression was primarily connected to i) cell envelope-associated processes, including proteins involved in exopolysaccharide synthesis and surface and membrane associated proteins, ii) to changes in growth and metabolic activities (C and N), including upregulation of nitrogenase and proteins involved in the oxidative pentose phosphate pathway and downregulation of Calvin cycle enzymes, and iii) to the dark, micro-aerobic conditions offered inside the *Gunnera* gland cells, including changes in relative phycobiliprotein concentrations. This is the first comprehensive analysis of proteins in the symbiotic state.

Cyanobacteria belonging to the filamentous and heterocystous genus *Nostoc* form symbiotic associations with a range of land plants from fungi to gymnosperms, but the genus *Gunnera* is the only flowering plant (angiosperm) (Rai et al. 2000). This is also the only plant symbiosis in which the cyanobacterium is located intracellularly and therefore shares similarities with the ancestral cyanobacterial symbiosis that led to the evolution of plant chloroplasts (Martin et al. 2002; Schopf 2000).

The symbiotic organs colonized by cyanobacteria in *Gunnera* plants are specialized stem glands (Bergman 2002; Bergman et al. 1992). When not yet infected, the glands secrete mucilage that attract *Nostoc* spp. and induce differentiation of vegetative filaments into motile hormogonia. The hormogonia enter the

gland interior through channels and subsequently infect certain *Gunnera* cells, in which they revert into nonmotile vegetative filaments (Johansson and Bergman 1992). Simultaneously, the differentiation of nitrogen-fixing heterocysts is profoundly stimulated (Rai et al. 2000), reflected in a severalfold increase in *hetR* expression (Wang et al. 2004), the product of which is required for heterocyst development (Buikema and Haselkorn 1991). Frequencies of up to 70 to 80% heterocysts have been observed in *Nostoc* spp. residing in older parts of the plant, as compared with 5 to 10% in young parts and in free-living *Nostoc* spp. The increase in heterocyst frequency is accompanied by increased nitrogen fixation activities, and most of the fixed nitrogen is released by the *Nostoc* cells and assimilated by the host plant (Silvester et al. 1996). This release is due to a reduced glutamine synthetase activity, the primary nitrogen assimilation enzyme in cyanobacteria (Söderbäck 1992). In return, the cyanobacteria are fed by fixed carbon from the plant, being photosynthetically inactive in the dark interior of the *Gunnera* glands (Black et al. 2002; Söderbäck and Bergman 1992). Other observed symbiotic modifications include enlargement of the *Nostoc* cell volume, interpreted as a blocked cell division possibly induced by the plant as a means of restricting growth of the cyanobacterium. The physiological adaptations are influenced by gland age (Söderbäck and Bergman 1992, 1993; Söderbäck et al. 1990). The highest frequency of heterocysts is restricted to older glands, and the expression of certain cyanobacterial genes, such as *hetR*, *ntcA*, and *nifH*, involved in cell differentiation and in nitrogen metabolism, show varied expression patterns along the gland age gradient (Wang et al. 2004).

The aim of the present study was to get a more holistic insight into mechanisms underlying the morphological and metabolic transformation of a free-living *Nostoc* sp. into a fully competent plant cyanobiont. The approach taken was to compare the expression patterns and the relative quantities of a large number of individual proteins in a fully adapted cyanobiont in *Gunnera manicata* versus in the corresponding free-living cyanobacterium. To achieve this aim, two-dimensional (2-D) gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS) combined with electrospray-tandem mass spectrometry (MS/MS) were used.

RESULTS AND DISCUSSION

Genetic identity of the cyanobiont and its free-living counterpart.

The cyanobacteria infecting *Gunnera* spp. are morphologically classified as belonging to the genus *Nostoc* (Bergman

2002; Rai et al. 2000). Previous studies have shown that most *Gunnera* plants are infected by single *Nostoc* strains (Guevara et al. 2002), although additional cyanobacterial strains may occur within the same plant (Nilsson et al. 2000). For comparing the proteomes of *Nostoc* filaments residing endosymbiotically in *Gunnera manicata* cells (cyanobionts) with those in the corresponding free-living *Nostoc* sp., it was crucial to verify the genetic identity of the strains. A polymerase chain reaction (PCR)-based DNA fingerprinting method, based on the presence of short tandemly repeated repetitive (STRR) sequences in the *Nostoc* genome, was used for this purpose. The cyanobacterium-specific STRR1A primers used give a resolution at the strain level (Rasmussen and Svenning 1998). The STRR-PCR fingerprints of freshly dissected *Nostoc* cyanobionts from two differently aged stem sections (young and old) of one *G. manicata* plant is illustrated in Figure 1 (A1 and A2). Approximately 35 glands were pooled at each age stage. As the DNA fingerprint patterns were identical at the two age stages, it was concluded that the one or more *Nostoc* strains were uniform throughout the plant. The free-living *Nostoc* strain AF, isolated from the symbiotic sample A1, also showed an identical fingerprint pattern (Fig. 1; lane AF). However, clearly different DNA fingerprints were obtained from freshly isolated *Nostoc* cells dissected from the smaller stoloniferous *G. magellanica* growing in the same greenhouse (Fig. 1; B1 and B2), from its free-living cultured counterpart (Fig. 1; BF), as well as from the cultured *Nostoc* strain PCC 9229 (originally isolated from *G. monoica*) (Fig. 1). Sequencing of the tRNA^{Leu} (UAA) intron of the cyanobiont of *G. manicata* (A1 and A2) and the free-living strain (AF), a sequence previously used to distinguish between cyanobacteria (Paulsruud and Lindblad 1998), produced identical sequences, thus supporting the identity between A1 and A2 and AF (data not shown).

Differential protein profiles in symbiotic and free-living *Nostoc* spp.

The symbiotic *Nostoc* material used for the extraction of total proteins (using a buffer containing urea, thiourea, dithiothreitol [DTT], and 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate [CHAPS], as described below) was composed of a mixture of equal amounts of the above-characterized freshly isolated samples, A1 and A2, therefore representing both young and older *Gunnera* gland cyanobionts. The heterocyst frequency in this material averaged about 30%. The protein extracts of the cyanobiont and the free-living counterpart AF were separated by 2-D gel electrophoresis covering a pH range of 4 to 7. As seen in Figure 2A and B, approximately 200 proteins from the cyanobiont and the free-living *Nostoc* sample (AF) could be visualized on SyproRuby-stained 2-D gels. The majority of the cyanobiont protein spots were also present in the free-living *Nostoc* sample, although some clear differences could be verified. Several proteins appeared to be down-regulated in the cyanobiont, while others were up-regulated.

A total of 56 protein spots that were repeatedly more than two times up-regulated (36 spots) or more than two times down-regulated (20 spots) were analyzed by MALDI-TOF/MS, and 30 of these were identified. Since MALDI-TOF/MS is dependent on a high degree of sequence similarity between the investigated protein and the database protein, even high quality MALDI-TOF spectra may be insufficient for identifying proteins from unsequenced organisms. Therefore, in order to expand the identification, eight of the unidentified proteins were further analyzed by MS/MS, which is less sensitive to differences in protein sequence (Shevchenko et al. 2001). This allowed the identification of three additional proteins, and one protein was identified by both MALDI-TOF/MS and MS/MS. Hence, a total of 33 of the analyzed spots were identified (Table

1, Fig. 2). Three of the proteins resolved into two different spots (g7-8, g9-10, and g21-22; Table 1), possibly due to posttranslational modifications of the proteins in question.

In an earlier study using *G. manicata* but with a different cyanobiont, 15 differentially expressed proteins were identified (data not shown). Seven of these were identified also in the present study and showed similar expression patterns. Five were not identified here, possibly because a narrower pH range was used in the previous study. These spots are, therefore, not marked on any gels but are still included in Table 1 (proteins g1 to gV). As the remaining three proteins did not change in abundance in the present study, these were not included here.

One reason for the difficulty in identifying additional proteins is probably that the symbiotic *Nostoc* strain examined is not identical to the cultivated and symbiotically competent *Nostoc* strain PCC 73102, of which the whole genome sequence is used as the basis for the protein identifications (available online from the Department of Energy, Joint Genome Institute). This is supported by the finding that all proteins isolated from heterocysts of *Nostoc* strain PCC 73102 could be identified (discussed below). The fact that more than 50% of the proteins examined were identified using peptide mass fingerprinting demonstrates a close relatedness between *Nostoc* strain PCC 73102 and the cyanobiont examined here and also points to the potential for using the proteomic approach to study nonsequenced cyanobionts in other natural plant symbioses. Using MS/MS in parallel increases both the confidence of identifications and the number of identified proteins.

Fig. 1. Short tandemly repeated repetitive polymerase chain reaction fingerprinting of symbiotic and free-living *Nostoc* strains. Lanes A1 and A2 represent symbiotic *Nostoc* cells freshly dissected from glands of two different age stages (young and old) of one *Gunnera manicata* plant. AF represents a cultured free-living strain isolated from A1. B1 and B2 represent symbiotic *Nostoc* cells freshly dissected from the smaller *G. magellanica*. BF is a cultured free-living strain isolated from *G. magellanica*. *Nostoc* PCC 9229 is a free-living strain isolated from *G. monoica* in 1992. M indicates the molecular weight standard.

Protein patterns in heterocysts versus vegetative cells.

Given the often severalfold elevated frequency of heterocysts in cyanobionts compared with free-living cyanobacteria, it was necessary to first compare the overall protein patterns in vegetative cells and heterocysts to estimate the contribution of heterocysts to the protein changes seen in the cyanobiont. The protein patterns obtained after separating proteins from a vegetative cell fraction and a heterocyst-enriched fraction (approximately 80% heterocysts) of the cultured *Nostoc* strain PCC 73102 were compared. As seen in Figure 3A and B, the majority of the proteins in the vegetative cells also appeared in the heterocysts. However, some proteins were down-regulated, a few were clearly up-regulated, and some were only present in the heterocyst protein fraction. In total, eight spots that were present only in the heterocyst were analyzed by MALDI-TOF/MS (Fig. 3; Table 2). As expected, proteins of the nitrogenase enzyme were exclusive to the heterocyst-enriched fraction. Five other proteins (h1 to h4 and h6) were associated with cell envelope functions, which is logical, as heterocysts have additional cell envelope layers, an outer polysaccharide layer, and an inner glycolipid layer, added to their original gram-negative cell wall (Wolk et al. 1994). One DNA-binding ferritin (h7) was also highly up-regulated in the heterocyst preparation. This protein was absent in vegetative (ammonia-grown) cultures but present in N-deficient (N₂-fixing) cultures (data not shown). This shows that this ferritin responds to nitrogen limitation, and indeed, this gene has an NtcA binding sequence at -113, a transcription factor influencing the expression of N-regulated genes in cyanobacteria (Flores and Herrero 2005). One of the enzymes specific for the oxidative pentose phosphate pathway, 6-phosphogluconate dehydrogenase (6PGD) (h8), was also up-regulated. It has previously been shown that this pathway has an important function in providing reductant for nitrogen fixation in heterocysts (Summers et al. 1995). It should be noted that the frequency of heterocysts in the *Gunnera* cyanobiont was less than half of that in the heterocyst-enriched fraction of *Nostoc* strain PCC 73102 examined.

Proteins affected in the cyanobiont.

As seen in Figure 2 and Table 2, several cyanobacterial proteins were affected by the symbiotic conditions prevailing in *Gunnera manicata*. Of the proteins identified, 23 were up-regulated in the cyanobiont in the plant while 10 were down-regulated.

Cell envelope and extracellular carbohydrates.

Four of the up-regulated proteins were surface- or membrane-associated (g3, g16, g26, gII; Table 1). One of these (g3) contained fasciclin-like repeats. A protein with such repeats was earlier identified to be highly up-shifted in the *Nostoc* cyanobiont of a lichen symbiosis and was suggested to have a role in symbiosis (Paulsrud and Lindblad 2002). However, as this protein was also up-regulated in heterocysts, as was protein g16, the high level of the fasciclins in symbiosis may rather be caused by the high heterocyst frequency. In the genome of symbiotically-competent *Nostoc* strain PCC 73102, the gene encoding protein gII is located just downstream of a sugar uptake operon. This operon was induced in *Nostoc* strain PCC 73102 when grown on fructose in darkness (data not shown), indicating that fructose may be the carbohydrate supplied by the host.

Three of the downregulated proteins are known to be involved in exopolysaccharide (EPS) and lipopolysaccharide (LPS) synthesis in bacteria. These were UDP-glucose 6-dehydrogenase (g21, 22) (Kereszt et al. 1998; Xue et al. 2004) and two phosphomannose-mutases (g27 and g30) (Bahat-Samet et al. 2004; Coyne et al. 1994). Such data are supported by examinations of the *Gunnera* cyanobiont and its free-living counterpart by light (Fig. 4) and transmission electron microscopy (Figs. 4E and 5). In symbiosis, the cyanobiont showed larger cells, more convoluted filaments, and a higher heterocyst frequency, while Alcian blue staining demonstrated that it produces considerably less EPS (Fig. 4, A compared with C). Moreover, the fibrillar nature of the heterocyst envelope and a thinner polysaccharide layer was evident in heterocysts of the cyanobiont after

Fig. 2. Protein patterns in extracts of **A**, freshly isolated symbiotic *Nostoc* cells of *Gunnera maincata* and **B**, its free-living counterpart *Nostoc* AF. Proteins were separated by two-dimensional gel electrophoresis, using a pH gradient of 4 to 7 and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Sypro Ruby.

Table 1. Proteins differentially expressed in symbiotic compared with free-living *Nostoc* strains

Gel ID ^b	Gene number	Annotation	Volume ^c	Mowse ^d	Matched ^e	% Seq cov ^f	Theoretical ^a		Observed	
							Mr	pI	Mr	pI
g1	ZP_00109717	Hypothetical protein	on	*73	8 of 27	19	44	4.1	43	4.0
g2	ZP_00108348	Akinete marker protein	28.9	91	7 of 16	24	34	4.7	37	4.6
g3	ZP_00108174	Secreted and surface protein containing fasciclin-like repeats	6.4	**96	7 of 11	21	59	5.0	55	4.5
g4	ZP_00107336	ATP synthase, beta subunit	2.0	130	10 of 18	32	52	5.0	50	4.9
g5	ZP_00111405	ATP synthase, alpha subunit	2.1	195	17 of 27	36	55	5.0	58	5.1
g6	ZP_00106350	Uncharacterized conserved protein	6.0	122	9 of 16	29	39	5.1	36	5.5
g7,8	ZP_00111829	Rhodanese related sulfur transferase	on	*44	4 of 27	22	22	4.8	25	4.8
g9,10	ZP_00109808	Mn-containing catalase	on	90	5 of 7	29	26	5.1	23	5.1
g11	ZP_00106529	DNA-binding ferritin-like protein	on	78	5 of 10	27	20	5.0	17	5.5
g12	ZP_00111427	Allophycocyanin beta chain	on	80	5 of 12	43	17	5.4	16	5.1
g13	ZP_00107296	Allophycocyanin beta chain	on	103	6 of 9	39	18	5.6	15	5.4
g14	ZP_00106785	Phycocyanin alpha chain	on	103	6 of 11	39	18	7.8	16	6.8
g15	ZP_00112380	Cysteine synthase	4.1	91	6 of 9	23	34	5.9	30	6.3
g16	ZP_00107013	Outer membrane protein	2.9	146	12 of 20	29	79	9.4	60	5.1
g17	ZP_00106110	Transketolase	2.6	80	7 of 18	18	73	5.8	77	5.9
g18	ZP_00111860	6-Phosphogluconate dehydrogenase	3.3	91	6 of 9	23	52	5.6	45	6.1
g19	ZP_00109568	DNA-binding ferritin-like protein	4.0	77	5 of 12	38	21	5.0	19	5.1
g20	ZP_0010511	Transcriptional regulator	0.36	71	7 of 17	17	56	6.0	58	4.7
g21, 22	ZP_00105906	Predicted UDP-glucose 6-dehydrogenase	0.04	139	9 of 11	29	51	5.4	45	5.4
g23	ZP_00109191	Phosphoribulokinase	0.26	132	8 of 10	38	39	5.4	40	5.6
g24	ZP_00106848	Phycocerythrin alpha chain	0.14	76	5 of 8	43	18	6.6	17	6.0
g25	AAA50768	Fructose-1,6-bisphosphatase	2.5	110	7 of 12	40	39	5.5	37	5.3
g26	ZP_00109067	Uncharacterized protein with a C-terminal OMP, S-layer homology	on	83	9 of 31	22	63	4.7	60	4.6
g27	ZP_00107278	Phosphomannomutase	0.23	83	6 of 10	19	52	5.2	50	5.1
g28	ZP_00109876	Glutaredoxin	0.30	111	7 of 12	39	27	5.1	26	5.0
g29	ZP_00105969	Hypothetical protein	off	***	nd	nd	67	5.1	59	5.3
g30	ZP_00109278	Phosphomannomutase	0.15	93	7 of 9	21	52	5.6	49	5.3
g31	ZP_00112137	NAD/NADP transhydrogenase alpha subunit	off	132	9 of 17	33	41	5.3	38	5.3
g32	ZP_00106446	Response regulator	5.6	93	7 of 9	35	26	5.7	25	5.7
g33	ZP_00112319	Nitrogenase reductase (NifH)	4.3	144	9 of 11	30	33	4.8	32	5.0
gI	ZP_00108634	Carbonic anhydrase	<0.5	80	6 of 20	34	26	6.0	24	5.3
gII	ZP_00106756	Uncharacterised protein, S-layer homology	>2	90	8 of 20	23	63	7.0	60	5.3
gIII	ZP_00111277	3-phosphoglycerate kinase	<0.5	177	13 of 23	42	42	5.1	42	5.0
gIV	ZP_00106143	Ketol-acid reductoisomerase	<0.5	142	9 of 14	38	36	5.3	36	5.2
gV	ZP_00108081	Vipp1 (thylacoid formation)	<0.5	113	12 of 18	35	29	5.1	34	5.2

^a Observed and the theoretical value, the observed being estimated from the two-dimensional gel and the theoretical calculated by the Mascot search engine. Mr indicates the molecular mass of the protein in kD, and pI indicates the isoelectric focusing point. Mr = the molecular mass of the protein in kD and pI = the isoelectric focusing point.

^b Proteins in this study are numbered g1 to g33. Proteins identified in a previous study but not observed in this study are numbered with roman numbers (gI to gV). Proteins identified in both studies appear in boldface type.

^c The volume ratio is the average relative abundance of the spots from three replica symbiotic gels divided by the average abundance on three replica free-living gels. Spots were chosen that were consistently up- or down-regulated more than two times on all replica gels. "On" indicates that the protein was only found on the gels of symbiotic *Nostoc*, and "off" only on those of free-living *Nostoc*.

^d Identifications are based on tandem mass spectrometry (indicated by *), sequence homology (**), or both (***). The matrix-assisted laser desorption/ionization-time of flight mass spectra of the spots that were identified as the same protein (g7-8, g9-10, and g21-22) were nearly identical and data are therefore presented for only one of the spots.

^e Matched peptides.

^f Percent of the predicted ORF that is covered by matching peptides.

Ruthenium red staining (compared in Figs. 4E and 5). LPS and EPS may have partly common biosynthetic pathways, and it has been speculated that the EPS layer of heterocysts may itself be an LPS (Huang et al. 2005). Furthermore, a protein (g20) containing the conserved LytR domain was down-regulated in the cyanobiont. Proteins containing this domain are transcriptional regulators involved in biosynthesis of extracellular polysaccharides, fimbriation, expression of exoproteins, including toxins, and in quorum sensing (Nikolskaya and Galperin 2002). However, proteins containing this domain have also been shown to be involved in cell division (Chatfield et al. 2005). The lower Mowse score of this protein (71) should be noted, and a confirmation of the identity and role of this protein is needed.

A reduced production of extracellular polysaccharides could be caused by differences in C/N ratios (Otero and Vincenzini 2004), the absence of light (Otero and Vincenzini 2003), or by

the microaerobic conditions prevailing inside the infected plant tissues (data not shown). For instance, the downregulation of the phosphomannomutase gene seen here has also been observed in *Pseudomonas aeruginosa* under low-oxygen conditions (Leitao and Sa-Correia 1997). The glycolipid layer of heterocysts is also known to decrease in thickness with decrease in oxygen concentration (Kangatharalingam et al. 1992). Modifications of the glycolipid or polysaccharide layer of heterocysts may be involved in the release of the nitrogen fixed (up to 90%) by the cyanobiont and may also explain the oxygen-sensitive nitrogen fixation of freshly isolated cyanobionts (Rai et al. 2000; Silvester et al. 1996).

Dark response.

The dark interior of the *Gunnera* glands is reflected in a modified phycobiliprotein composition of the cyanobiont. As seen in Figure 2 and Table 1, allophycocyanin (g12 and g13)

Fig. 3. Protein patterns in extracts of **A**, heterocyst enrichment and **B**, vegetative cells of *Nostoc* strain PCC 73102. Proteins were separated by 2-dimensional gel electrophoresis using a pH gradient of 4 to 7 and 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Sypro Ruby.

Table 2. Proteins up-regulated in heterocysts of *Nostoc* strain PCC 73102

Gel ID	Gene number	Annotation	Mowse score	Matched peptides	% Seq cov ^b	Theoretical ^a		Observed	
						Mr	pI	Mr	pI
h1	ZP_00108174	Secreted and surface protein containing fasciclin-like repeats	90	8 of 19	2	59	5.0	60	4.6
h2	ZP_00106112	Autotransporter adhesin	96	9 of 27	28	62	4.9	50	4.5
h3	ZP_00107013	Outer membrane protein	186	12 of 13	30	79	9.4	50	5.4
h4	ZP_00110600	Periplasmic protein involved in polysaccharide export	112	9 of 17	25	51	7.8	55	5.9
h5	ZP_00112319	Nitrogenase reductase (NifH)	83	8 of 13	22	33	4.8	37	4.9
h6	ZP_00108185	Hypothetical protein S-layer	259	18 of 26	52	60	4.8	50	4.7
h7	ZP_00109568	DNA-binding ferritin	105	8 of 18	43	21	5.0	22	4.9
h8	ZP_00111860	6-Phosphogluconate dehydrogenase	116	8 of 11	30	58	5.5	60	5.7

^a The observed value was estimated from the two-dimensional gel. The theoretical value was calculated by the Mascot search engine. Mr indicates the molecular mass of the protein in kD, and pI indicates the isoelectric focusing point.

^b Percent of the predicted ORF that is covered by matching peptides.

and phycocyanin (g14) levels were up-regulated, while phycoerythrin was down-regulated as compared with that in the free-living counterpart. The bluer pigmentation of the cyanobiont was obvious using light microscopy (Fig. 4D), and spectrophotometric absorbance wavelength scanning showed a relatively higher absorption around 620 nm (phycocyanin) and a lower at 560 (phycoerythrin) (Fig. 6A). After normalizing these levels to chlorophyll a concentrations, the free-living *Nostoc* strain had about equal levels of the pigments, while the cyanobiont phycoerythrin level was only about half that of phycocyanin and allophycocyanin (Fig. 6B). A decrease in phycoerythrin levels was also observed for *Nostoc* strain Mac grown in darkness, a change also expected for chromatic adaptation to red light (Austin et al. 1996). The downregulation of Vipp1 (gV in the cyanobiont [Table 1]), a protein needed for thylakoid membrane synthesis in *Synechocystis* sp. strain PCC 6803 (Westphal et al. 2001), may also be a reflection of the dark interior of the *Gunnera* gland tissues.

Energy, nitrogen, and carbon metabolism.

As seen in Table 1, the dinitrogenase reductase (NifH) (g33) level was approximately four times higher in the cyanobiont than in the free-living counterpart, which is in accordance with previous studies using immuno-cytochemistry and the higher

rates of nitrogen fixation observed in *Gunnera* symbioses (Söderbäck et al. 1990). Since nitrogen fixation is a highly energy-demanding process, mechanisms involved in supplying ATP are central to symbiosis. This may explain the high levels of ATP synthase detected (Fig. 2 and Table 1; g4 and g5) and suggests that oxidative phosphorylation is crucial for ATP production in the cyanobiont.

6PGD (Fig. 1 and Table 1; g18) is one of the key enzymes of the oxidative pentose phosphate (OPP) pathway. The fact that this enzyme was up-regulated in the cyanobiont stresses the importance of this pathway for oxidizing plant-supplied carbohydrates. This is also supported by previous reports on dark heterotrophic growth of *Nostoc* sp strain ATCC 29133 (Summers et al. 1995). As the same protein was up-regulated in heterocysts (Table 2), the upregulation in the cyanobiont may be associated with the higher heterocyst frequency in symbiosis. Indeed, OPP is the main pathway for producing reducing equivalents for nitrogen fixation (Wolk et al. 1994). Alternatively, the upregulation of 6PGD may also be associated with the reduced growth rate in symbiosis, since expression of 6PGD in *Synechococcus* sp. strain PCC 7942 is known to be growth-phase dependent (Broedel and Wolf 1991).

Glucose-6-phosphate dehydrogenase (G6PD) is also specific for the OPP pathway. The gene encoding G6PD is located in an operon containing genes encoding transaldolase and fructose-1,6-bisphosphatase (FBP) (Fig. 1 and Table 1; g25), and FBP is known to be up-regulated under diazotrophic conditions (Summers and Meeks 1996). There are two types of FBP in cyanobacteria, the commonly expressed FBP with sedoheptulose-1,7-bisphosphatase (SBP) activity and the FBP of the operon mentioned above (Tamoi et al. 1998). Our data show that FBP of the G6PD operon is up-regulated in the cyanobiont and may therefore have a specific function for the OPP pathway in heterocysts, in combination with dark heterotrophic growth conditions, or both. This FBP may be replaced by the FBP and SBP under photoautotrophic conditions or in vegetative cells. Transketolase (g17) is an enzyme common for the OPP pathway and the Calvin cycle. As this enzyme is up-regulated in the cyanobiont while the Calvin cycle is inactive (Söderbäck and Bergman 1992), also reflected in the down-regulation of one of the key enzymes of the cycle, phosphoribulokinase (g23), the importance of the OPP pathway under symbiotic conditions is again stressed.

Intermediates of glycolysis are, however, also present in glucose-supplied gland tissue (Black et al. 2002) suggesting that the OPP pathway is not the only pathway active in catabolizing sugars in the *Gunnera* symbiosis. Phosphoglycerate kinase (gIII) is common for both glycolysis and the Calvin cycle. The downregulation detected here may therefore reflect a reduced carbon flow through glycolysis or an inactive Calvin cycle. Downregulation of certain other metabolic enzymes, such as NAD/NADP transhydrogenase (kb31) and ketol-acid reductoisomerase (gIV), may be related to the reduced growth rate, a switch to a low-oxygen adapted metabolism, or to the reduced percentage of vegetative cells.

Being photosynthetically inactive, the upshift in carbonic anhydrase (Table 1, gI) is not likely associated with providing high concentrations of CO₂ at Rubisco and suggests one or more still-unknown roles for this enzyme in cyanobacteria. Indeed, the most homologous proteins to the gI protein of the cyanobiont are found in nonphotosynthetic bacteria, in which carbonic anhydrases are involved in heterotrophic growth and fermentation (Smith and Ferry 2000). The enzyme is also up-regulated 24-fold in *Salmonella typhimurium* inside its host cell (a macrophage), and a role in intracellular growth and survival has been suggested (Valdivia and Falkow 1997).

Fig. 4. Light microscopic images of Alcian blue-stained exopolysaccharides (EPS) of the **A**, cultured and **C**, symbiotic *Nostoc* (*Gunnera manicata*) cells demonstrating more pronounced EPS layers (thick arrows) in the free-living stage. **B** and **D**, Nonstained controls. **E**, transmission electron micrograph of a ruthenium red-stained free-living *Nostoc* cell showing the fibrillar nature of the EPS layer (arrow). Note the higher heterocyst (H) frequency in the cyanobiont. Scale bars represent 15 μ m (**A** to **D**) and 1 μ m (**E**).

Stress response proteins.

When endosymbiotic, the growth rate of the cyanobiont is reduced, and it has been speculated that this is regulated by the host. The mechanism is unknown, but one possibility is via a restricted nutrient availability in symbiosis. This is supported by the finding that ferritins (g11 and g19) were up-regulated in the cyanobiont. Members of the Dps family of proteins, including the DpsA up-shifted in the cyanobiont, are ferritins that bind and protect DNA under conditions of nutrient and oxidative stress (Pena and Bullerjahn 1995). One of the upregulated ferritins (g19) was also up-regulated in the heterocyst preparation, indicating a heterocyst-specific function of g19.

Cysteine synthase (g15) catalyzes the formation of the essential amino acid cysteine. This enzyme is known to be involved in stress response and starvation survival mechanisms in the human *Staphylococcus* pathogen (Lithgow et al. 2004). In sulfur-limited *Bacillus subtilis*, the expression of this enzyme is also up-regulated (Coppee et al. 2001). Upregulation of cysteine synthase in the *Gunnera* cyanobiont may therefore reflect a low-nutrient environment in the *Gunnera* glands. This may also be the reason for the upregulation of the rhodanese-related sulfurtransferase (g7, 8) in the cyanobiont. The function of these enzymes is unclear, but they have been reported to be involved in biosynthesis, repair of Fe-S centers of proteins, or

Fig. 5. Transmission electron micrographs of heterocysts of **A**, free-living *Nostoc* strain PCC 73102 and **B**, the symbiotic *Nostoc* strain occupying *Gunnera manicata* cells. Note the thinner outer polysaccharide layer (arrows) of the heterocysts in symbiotic *Nostoc* cells as well as the larger cell size. Bar represents 0.8 μm .

Fig. 6. Phycobiliproteins in free-living and symbiotic *Nostoc* cells. **A**, Absorption spectra of soluble phycobiliproteins of free-living *Nostoc* strain AF, isolated from the *Gunnera manicata* symbiosis and freshly extracted *Nostoc* cyanobiont from the same symbiosis. The absorption at 560 nm, corresponding to phycoerythrin (PE), relative to the absorption at 620 nm, corresponding to phycocyanin (PC), is lower in symbiotic than in free-living *Nostoc* cells. **B**, When calculating the phycobiliprotein concentration and normalizing it to the chlorophyll a concentration, it is obvious that mainly PE levels decrease in symbiotic *Nostoc* cells. AP = allophycocyanin.

both, detoxification mechanisms of the reactive oxygen species, and in assimilatory sulfate reduction (Melino et al. 2004).

Catalases are usually involved in protection against oxidative stress. Since the environment inside the glands is microaerobic, the upregulation of the Mn-catalase (g9, 10) is unexpected. However, in *Rhizobium*-legume symbioses, both bacterial catalases and a peroxiredoxin have been reported to be up-regulated despite low-oxygen conditions (Dombrecht et al. 2005). The reason for this upregulation was suggested to be a combination of nutrient and carbon limitation, stationary phase-like growth, a high need to protect nitrogenase, and a high respiration. Heterocysts of free-living cyanobacteria also show a high specific catalase activity (Fay 1992). Another protein involved in the defense against oxidative stress, glutaredoxin (g28), was, on the other hand, down-regulated in the cyanobiont, and it therefore seems as if different sets of stress-response proteins are expressed under symbiotic versus free-living conditions. Alternatively, as glutaredoxin may also be involved in the formation of deoxyribonucleotides for DNA synthesis (Fernandes and Holmgren 2004), the downregulation of this protein in the cyanobiont may be related to a reduced rate of cell division.

Other proteins.

One of the proteins (g32) up-regulated in the cyanobiont was identified as a homolog to the *orrA* response regulator in *Anabaena* sp. strain PCC 7120 (Schwartz et al. 1998), the expression being stimulated in the presence of various osmotica such as NaCl or sucrose. An upregulation of this protein in symbiosis may be due to a higher osmotic pressure caused by host-supplied carbohydrates.

As the 'akinetete marker' protein (g2), previously found to be highly expressed in akinetes of *Anabaena variabilis* (Zhou and Wolk 2002), was both up-shifted in the akinete-free cyanobiont of *Gunnera* and down-regulated in hormogonia of *Nostoc* strain PCC 73102 (Klint 2003), the function of this protein needs to be redefined. It should also be noted that four homologous copies are present in the *Nostoc* strain PCC 73102 genome.

Overall perspective.

When entering *Gunnera* cells, the normally photoautotrophic genus *Nostoc* is exposed to a strikingly different environment, such as being intracellular, in darkness, and microaerobiosis. In addition to having to adapt to this environment, *Nostoc* strains are influenced by the plant in certain ways, reflected in the high heterocyst frequency, the export of fixed nitrogen, and the reduced growth and cell division rates. Our study shows that despite these physiological differences, most of the proteins present in symbiosis are also present in the free-living state, indicating that most cellular functions remain largely intact in symbiosis. This is also verified by the ease with which *Nostoc* strains, in contrast to, for instance, the *Azolla* cyanobiont (Rai et al. 2000), resume growth as free-living after being isolated from symbiotic plant tissues.

However, vital adaptations of the cyanobiont that were of symbiotic relevance were observed. The upshift in the key symbiotic process, nitrogen fixation, is clearly reflected in the cyanobiont protein pattern, as is the mechanism by which this process is supplied with energy. An upregulation of the OPP pathway was apparent, while the Calvin cycle, as expected, was down-regulated. A metabolic focus on heterocyst-related metabolism and function is also reflected in the fact that most of the proteins up-regulated in the heterocysts were also up-regulated in the cyanobiont. Indeed, the symbiotic conditions offered in planta, i.e., heterotrophy and microaerobiosis, are similar to those already prevailing in heterocysts. One may therefore speculate that adapting to a symbiotic life involves mechanisms already developed for heterocysts.

Several proteins differentially expressed in the cyanobiont were membrane- or surface-related, implicating surface adaptations as being of particular importance for the establishment of the symbiosis and for nutrient exchange, which also is in accordance with our earlier observations (Klint 2003; Liaimer 2002). Downregulation of certain proteins may, furthermore, be related to potential plant- or microaerobiosis-induced changes of the heterocyst cell wall and thereby to the release of ammonia.

A third group of proteins affected were nutrient and oxidative stress related, indicating that the cyanobiont is kept nutrient limited and that this may be a mechanism by which the host regulates the growth rate of the cyanobiont.

In conclusion, our results demonstrate that the proteomic approach is an excellent tool to discover novel and nonpredictable changes and adaptive processes in such complex associations as the one between cyanobacteria and plants. Although the endosymbiotic *Nostoc* strain investigated has not been sequenced, a high percentage of proteins were identified based on the sequenced genome of *Nostoc* strain PCC 73102. As this genome is suggested to contain more than 7,000 open reading frames (Meeks et al. 2001), the proteins identified here clearly represent a fraction of all proteins and the full potential of symbiotic-specific plant-induced regulation is still to be identified.

MATERIALS AND METHODS

Organisms and growth conditions.

G. manicata Linden plants were grown in the greenhouse at the Department of Botany at Stockholm University (Stockholm, Sweden) at 15°C. Cyanobacteria isolated from one of these plants (discussed below) and *Nostoc* strain PCC 73102 were cultivated in nitrogen-depleted BG11₀ medium (Stanier et al. 1971) at constant light (18 $\mu\text{mol photons m}^{-2} \text{ s}^{-2}$) and shaking at 25°C. For obtaining heterocyst-free *Nostoc* strain PCC 73102, this strain was cultivated in BG11₀ with the addition of 2.5 mM NH_4Cl and was buffered with 5 mM MOPS (morpholinepropanesulfonic acid), pH 7.8.

Isolation of symbiotic *Nostoc* strains from *G. manicata*.

The *G. manicata* plant stem containing the *Nostoc*-infected glands was cut free of leaves and other material, was washed with 70% ethanol followed by sterile water, and then, was divided into two sections, the upper and younger part close to the growing plant apex and the lower, older part. Approximately 35 *Nostoc*-infected glands from each stem section were cut out using a sterile scalpel, and the *Nostoc* cells were excised into ice-cold buffer A (1% [vol/vol] Tween 20, 15 mM MgCl_2 , and 2% [wt/vol] polyvinylpyrrolidone). The cells were then homogenized with a plastic pestle in a microcentrifuge tube and were allowed to sediment. The supernatant was kept, and the pellet was resuspended in buffer A and allowed to sediment again. This was repeated three times, after which the supernatants were pooled and twice centrifuged for 2 min. at $320 \times g$. The cells were then dissolved in 2 ml of buffer A and were placed on top of 80% Percoll (Amersham Biosciences, Uppsala, Sweden) and centrifuged for 20 min at $800 \times g$ at 4°C. The cells stayed on top of the Percoll while *Gunnera* starch grains sedimented. The excised *Nostoc* cells from all glands of each stem section were pooled and mixed into two samples, A1 and A2 (young and old). Microscopic observation showed that the suspensions were dominated by *Nostoc* cells, and only a small proportion of plant cell debris was present. The heterocyst frequency was also estimated by light microscopy. The cell suspensions were transferred to microcentrifuge tubes and centrifuged ($15,800 \times g$), and the pellets were frozen at -20°C . Ap-

proximately 50 µl of the A1 suspension was plated on a BG11₀ agar (1.5%) plate and was maintained under the conditions given above. After a few weeks, colonies developed and were transferred to liquid BG11₀ medium. A free-living *Nostoc* culture obtained in this way was named AF. From this culture, approximately the same amount of cells was harvested from the AF culture as from the symbiotic *Nostoc* culture (50 mg wet weight).

Isolation of heterocysts.

Heterocysts were isolated using a method based on that of Smith and associates (1988). *Nostoc* strain PCC 73102 was grown in BG11₀ medium under continuous light and stirring in BG11₀ batch cultures of 1 liter. Filaments were collected in the exponential phase by centrifugation, and the pellet was washed twice in buffer A (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1 mM MgCl₂). Lysozyme (1 mg/ml) was added, and the cells were incubated at 37°C for 1 h. To fully disrupt the vegetative cells, the suspensions were also exposed to sonication at 70% intensity for 15 s and were washed three to four times with buffer A, using a centrifuge speed of 250 × *g*. This usually resulted in approximately 80% heterocyst enrichment. These heterocyst-enriched pellets were frozen, and proteins were extracted as described below. A pure vegetative cell sample was obtained by culturing *Nostoc* strain PCC 73102 in BG11₀ medium supplemented with NH₄Cl, as described above.

Protein extraction.

The cyanobacterial pellets (freshly extracted and cultured cyanobiont and heterocyst enrichments and vegetative cells of *Nostoc* strain PCC 73102) were frozen in liquid nitrogen, a few grains of acid-washed sand were added, and the cells were ground with a plastic pestle. A total of 200-µl of sample buffer containing 8 M urea, 2 M thiourea, 2.8% (wt/vol) DTT, protease inhibitor (1 tablet to 10 ml Complete mini; Roche Diagnostics, Mannheim, Germany), and 2% (wt/vol) CHAPS was then added. The samples were then frozen and thawed three more times in liquid nitrogen. Four samples were pooled, resulting in a total sample volume of 800 µl and were sonicated five times for 3 s at 70% intensity with 30-s intervals on ice to avoid heating. The samples were then centrifuged at 15,800 × *g* at 4°C for 15 min to remove remaining cell debris. The supernatant was collected and kept at -20°C until use. The protein concentration in the samples was determined using the RC DC kit (BioRad, Hercules, CA, U.S.A.). Proteins were also extracted from the noninfected *Gunnera* tissue surrounding the symbiotic glands, using the same procedure.

2-D gel electrophoresis.

Isoelectric focusing of the proteins was performed using the IPGphor system (Amersham Biosciences). Immobiline gel strips (18 cm, pH 4 to 7) were rehydrated overnight in the same buffer as that used for protein extraction, with the addition of 0.5% immobilized pH gradient buffer. Protein extract containing 150 µg of protein was applied to the gel strip by using application cups located at the acidic end of the strip. The proteins were separated by applying voltage as follows: 500 V for 30 min, 1,500 V for 1.5 h, and 8,000 V until reaching a total of 75,000 Vh. The strips were then incubated in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% [vol/vol] 87% glycerol [vol/vol], 2% [wt/vol] SDS) with 1% (wt/vol) DTT added for 15 min, and subsequently, in equilibration buffer with 4% (wt/vol) iodoacetamide added. The proteins were then separated according to size in a 10% acrylamide gel run at 10 V per gel for 30 min and then for 25 V per gel for approximately 4.5 h. The gels were stained with SyproRuby (BioRad) according to the manufacturer's instructions, and a

Typhoon 8600 laser scanner was used to visualize the gels (Amersham Biosciences). To obtain quantitative estimates of the protein spots, the scanned 2-D protein patterns were analyzed by PDQuest software (BioRad). Three replica gels of each treatment were included in the analysis, and protein spots were only further analyzed if a consistent change in abundance of two times or more was observed.

In-gel digestion and MS.

In-gel tryptic digestion was performed according to Gharahdaghi and associates (1999). Mass spectra were obtained using a Voyager-DE STR mass spectrometer operating in reflective mode (MALDI-TOF; Applied Biosystems, Foster City, CA, U.S.A.). Two-point internal calibration of the peptide mass fingerprints was achieved using internal trypsin autolysis peptides and MoverZ software, available online from Genomic Solutions (Ann Arbor, MI, U.S.A.). The obtained peak lists were compared with databases, using the Mascot search engine available online from Matrix Science (Boston). NCBIInr was chosen as sequence database, taxonomy was set to all organisms, a mass tolerance was set to 30 ppm, fixed modifications was set to alkylation of cysteine by carbamidomethylation, and variable modifications to oxidation of methionine. Only peptides with a probability-based Mowse score above 76, indicating a significance level of $P < 0.05$, were considered to be successful identifications.

Electrospray-MS/MS experiments were carried out using a Micromass Q-ToF (Waters, Milford, MA, U.S.A.). Prior to analysis, salts and contaminants were removed from the samples by ZipTip purification and the peptides were eluted in water/acetonitrile (1:1) with 0.1% of formic acid. Samples (1 to 3 µl) were loaded into a nanospray tip (Proxeon, Odense, Denmark). The capillary voltage was kept at 1,200 to 1,300 V and the cone voltage at 45. Argon was used as collision gas at an inlet pressure of 0.7 Bar, and fragmentation was achieved by increasing the collision energy to 20 to 35. Doubly charged ions were selected for fragmentation and tandem mass spectra were acquired for 1 to 10 min. The spectra were processed with MassLynx MaxEnt 3 before database searches in NCBIInr utilizing Mascot. Proteins g3 and g29 were not identified by this procedure and were therefore sequenced de novo, using the processed tandem mass spectra and the BioLynx peptide sequencing tool (Micromass). All suggested sequences for all fragmented peptides were submitted to MS-Homology from ProteinProspector, available online from the University of California, San Francisco.

DNA extraction and PCR amplification.

DNA was extracted, using the GenElute plant genomic DNA miniprep kit (Sigma-Aldrich, St. Louis), from the following samples: symbiotic *Nostoc* cells freshly excised from two different *G. manicata* stem sections described above and denoted A1 and A2; the free-living *Nostoc* culture AF from the same stem glands described above; symbiotic *Nostoc* cells excised from the small stoloniferous *G. magellanica* (B1 and B2) from the same greenhouse (Department of Botany, Stockholm University); another free-living *Nostoc* culture previously isolated from *G. magellanica* (*Nostoc* BF); axenic culture of *Nostoc* strain PCC 9229 (originally isolated by E. Söderbäck from *Gunnera monoica* in New Zealand).

The oligonucleotide STRR1A (5'-CCARTCCCCARTCCCC-3'), corresponding to a short tandemly repeated repetitive (STRR) sequence present in the genome of heterocystous cyanobacteria (Rasmussen and Svenning 1998), was used as a primer in the PCR to obtain DNA fingerprints from these cyanobacteria. The PCR reaction volume was 25 µl, and each reaction contained 1 U polymerase (DynaZyme, Finnzymes, Oy,

Finland), 1× buffer (supplied with the enzyme), 1.25 mM of each dNTP, 50 pmol STRR1A primer, and 50 ng of DNA template. PCR reactions were run under the following conditions: 1 cycle at 95°C for 6 min, 40 cycles at 94°C for 1 min, 56°C for 1 min, 65°C for 5 min, 1 cycle at 65°C for 16 min, and a final step at 4°C. Amplification products (10 µl) were separated in 1.5% agarose gels. The gels were stained with ethidium bromide and were visualized by UV light and recorded with a CCD video camera (VC-XC-75CE, Sony, Tokyo).

Sequencing of the tRNA^{Leu} (UAA) intron.

The tRNA^{Leu} (UAA) intron was amplified by a nested PCR reaction with the outer primers A and C as described by Paulsrud and Lindblad (1998) and the inner primers TL25 and TL23 as described by Biniszkiwicz and associates (1994). As template, 1 µl of the same DNA as above was used in the first PCR reaction. The PCR products were purified using a commercial kit (Quiaquick PCR purification kit; Qiagen GmbH, Hilden, Germany) and the DNA fragments sequenced by DNA Technology (Aarhus, Denmark). The sequences were edited and compared using the BioEdit software, available online from North Carolina State University.

Light and transmission electron microscopy.

To analyze extracellular carbohydrates, cyanobionts freshly extracted from *G. manicata* glands and its free-living counterpart *Nostoc* strain AF were stained with 1% aqueous solution of Alcian blue for 40 s, were washed with deionized water, and were examined using light microscopy (Olympus BX60, Olympus, Tokyo). To examine cell wall structures and extracellular carbohydrates in more detail, the same samples were stained with 15% Ruthenium red for 1 h, followed by immobilization of cell pellets in 2% agar. Agar blocks were then dehydrated through an ethanol series up to 100% and embedded in Epon 812 (TAAB). Ultrathin sections on gold grids were examined using transmission electron microscopy (Zeiss EM106, Oberkochen, Germany) operating at 60 kV.

Chlorophyll and phycobiliprotein determination.

Phycobiliproteins were extracted from the cyanobacterial samples by the same procedure given above for total protein extractions, except that the sample buffer was substituted with a 20 mM sodium acetate buffer. The absorbance spectra of the extracts were scanned using spectroscopy (Ultrospec 3000, Pharmacia Biotech, Uppsala, Sweden) within a range of 400 to 750 nm. The phycobiliprotein concentrations were calculated using equations given by Tandeau de Marsac and Houmard (1988). The pellet remaining after centrifugation was incubated on ice in darkness for 1 h in methanol to extract chlorophyll a, and the absorbance of the supernatant was measured at 665 nm.

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AUTHOR RECOMMENDED INTERNET RESOURCES

Department of Energy Joint Genome Initiative website: www.jgi.doe.gov
 Genomic Solutions website: www.genomicsolutions.com
 Matrix Science Mascot website: www.matrixscience.com
 National Center for Biotechnology Information website: www.ncbi.nlm.nih.gov
 North Carolina State University, BioEdit website: www.mbio.ncsu.edu/BioEdit/bioedit.html
 University of California San Francisco, MS-Homology, ProteinProspector website: prospector.ucsf.edu