Arabinogalactan Proteins Occur in the Free-Living Cyanobacterium Genus *Nostoc* and in Plant–*Nostoc* Symbioses

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Arabinogalactan proteins (AGP) are a diverse family of proteoglycans associated with the cell surfaces of plants. AGP have been implicated in a wide variety of plant cell processes, including signaling in symbioses. This study investigates the existence of putative AGP in free-living cyanobacterial cultures of the nitrogen-fixing, filamentous cyanobacteria *Nostoc punctiforme* and *Nostoc* sp. strain LBG1 and at the symbiotic interface in the symbioses between *Nostoc* spp. and two host plants, the angiosperm *Gunnera manicata* (in which the cyanobacterium is intracellular) and the liverwort *Blasia pusilla* (in which the cyanobacterium is extracellular). Enzyme-linked immunosorbent assay, immunoblotting, and immunofluorescence analyses demonstrated that three AGP glycan epitopes (recognized by monoclonal antibodies LM14, MAC207, and LM2) are present in free-living *Nostoc* cyanobacterial species. The same three AGP glycan epitopes are present at the *Gunnera*–*Nostoc* symbiotic interface and the LM2 epitope is detected during the establishment of the *Blasia*–*Nostoc* symbiosis. Bioinformatic analysis of the *N. punctiforme* genome identified five putative AGP core proteins that are representative of AGP classes found in plants. These results suggest a possible involvement of AGP in cyanobacterial–plant symbioses and are also suggestive of a cyanobacterial origin of AGP.

Cyanobacteria are known to enter into a wide variety of symbioses (Bergman et al. 2008), including those with the water fern genus *Azolla* (Lechno-Yossef and Nierzwicki-Bauer 2002), lichens (Rikkinen 2002), the fungus genus *Geosiphon* (Kluge et al. 2002), hornworts, and liverworts, including *Anthoceros punctatus* and *Blasia pusilla* (Adams and Duggan 2008, 2011) and cycads (Costa and Lindblad 2002). Their ability to fix atmospheric nitrogen is often utilized in these relationships, providing their host with a source of combined nitrogen. Many cyanobacterial species capable of nitrogen fixation do so via specialized cells, known as heterocysts, which have a thickened cell wall and provide a microaerobic environment for the functioning of the highly oxygen-sensitive enzyme nitrogenase, which is responsible for the conversion of N₂ to NH₃. Heterocyst structure and function have been recently reviewed (Flores and Herrero 2010).

The symbiosis between the cyanobacteria of the genus *Nostoc* and the angiosperm genus *Gunnera* is unique because the cya-

nobiont forms colonies within plant cells in specialized gland structures at the base of each leaf petiole (Bergman 2002). The relationship is mutually beneficial, the plant receiving fixed nitrogen from the bacterium (Silvester and Smith 1969; Silvester et al. 1996) and the bacterium receiving fixed carbon from the plant (Black et al. 2002), possibly in the form of fructose (Ekman et al. 2006). The relationship is facultative, in that both the plant and the bacterium may be cultured separately (Silvester and McNamara 1976) although all species of *Gunnera* investigated in the wild contain *Nostoc* spp. as an intracellular symbiont (Osborne et al. 1991).

Gunnera spp. exert a high level of selectivity over the bacterial partners; only *Nostoc* spp. are found as symbionts, and only those strains capable of high levels of differentiation into hormogonia, which are specialized motile filaments that lack heterocysts and which are the infective agents in cyanobacteria–plant symbioses (Adams and Duggan 2011; Bonnett and Silvester 1981). Nonetheless, the ability to develop heterocysts and hormogonia is not enough to guarantee symbiotic competence—some strains are capable of both yet still do not generate stable symbioses with *Gunnera* spp. (Bonnett and Silvester 1981; Johansson and Bergman 1994). A second symbiosis studied in the work presented here is that between *Nostoc* spp. and the liverwort *B. pusilla* in which, unlike *Gunnera* spp., the cyanobiont does not enter the cells of the host plant but, instead, is contained within dome-shaped structures, known as auricles, on the ventral surface of the thallus (Adams 2002; Adams and Duggan 2008, 2011). The cyanobiont occupies the space between the auricle wall and the central slime papilla; an outer papilla is found adjacent but external to the auricle.

Arabinogalactan proteins (AGP) are a very diverse group of proteoglycans generally found associated with plant cell surfaces (Ellis et al. 2010; Nothnagel 1997; Seifert and Roberts 2007; Showalter 2001). They are rich in hydroxyproline and are highly glycosylated, with approximately 90% of the total molecular mass (usually between 60 and 300 kDa) (Nothnagel 1997) being attributed to the carbohydrate portion of the molecule (Showalter 1993). The glycan components of plant AGP are highly heterogeneous and contain arabinose and galactose along with rhamnose, glucuronic acid, and other sugars. The AGP glycan is linked by *O*-glycosylation to a small polypeptide core which generally constitutes less than 10% of the molecular mass (Ellis et al. 2010; Gaspar et al. 2001). The most abundant amino acids in the polypeptide core are proline (hydroxyproline), alanine, serine, threonine (collectively termed PAST) (Schultz et al. 2000).

AGP are divided into several families based on their protein cores (Gaspar et al. 2001; Seifert and Roberts 2007). Classical AGP core proteins consist of a region rich in PAST along with

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an N-terminal signal peptide and a hydrophobic C-terminal domain signal for the addition of a GPI anchor (Showalter 2001). Arabinogalactan (AG) peptides are a subset of the classical AGP—they consist of the same three regions but their PAST-rich region is truncated, and the entire peptide is typically less than 75 amino acids in length (Schultz et al. 2002). The fasciclin-like AGP (FLA) are a group of nonclassical AGP that differ from the classical AGP only in that they contain conserved fasciclin domains within the mature peptide (Faik et al. 2006; Johnson et al. 2003).

Some prokaryotes, such as mycobacteria, do have an AG component in cell envelopes along with mycolic acid and a peptidoglycan complex (or, confusingly, the mycolyl AG-peptidoglycan complex), which is covalently linked to their cell membrane (Crick et al. 2001). It should be noted that, whereas this fraction of the mycobacterium cell membrane shares the designation “AG” with the plant AGP, these are very different structures, with the arabinose being D-arabinose (whereas in plant AG the arabinose is largely L-arabinose). Hence, although there may be a designative link between the names of plant and bacterial AG, there is no evidence of the two being structurally or developmentally linked.

AGP have been implicated in several processes in plant cells: cell division, cell expansion, programmed cell death, and intercellular signaling, including plant-microbe interactions (Seifert and Roberts 2007). Endoglycanase enzymes may cleave off short oligosaccharides from the extended AGP structure, and it has been proposed that these may act as long-range signaling molecules, because they would be small and soluble, making them far more mobile than the entire AGP (Seifert and Roberts, 2007; van Hengel et al. 2001). This would allow AGP to have an effect on cell processes within and between cells and, in part, explain their wide-ranging potential roles. AGP have been shown to be present at the symbiotic interface in the *Frankia–Alnus* symbiosis (Berry et al. 2002), as well as being involved in the symbiosis between the arbuscular mycorrhizal fungus *Glomus intraradices* and the legume *Medicago truncatula* (Schultz and Harrison 2008).

Previous observations have raised the possibility that AGP may play a role in the *Gunnera–Nostoc* symbiosis (Rasmussen et al. 1996; Snogerup 1997) and plant–cyanobacterial symbioses in general. To study this further, we have made use of sets of monoclonal antibodies, each with a different binding site within the complex glycan structures of AGP, and which are used to investigate AGP occurrence at plant cell surfaces (Berry et al. 2002; Knox 2008; Lee et al. 2005). Here, we report the presence of specific AGP glycan epitopes in both free-living partners of the *Gunnera–Nostoc* and *Blasia–Nostoc* symbioses and at the interfaces between host cells and cyanobacteria. Moreover, we provide evidence from the genome of *Nostoc punctiforme* for the presence of putative AGP core proteins representative of the classical AGP, AG peptide, and FLA classes of AGP protein structures found in plants.

RESULTS

Free-living cyanobacterial strains express AGP epitopes.

To lay the foundation for an analysis of AGP in plant–cyanobacterial symbioses, the presence of AGP epitopes was assessed in two free-living and symbiotically competent *Nostoc* strains and their growth media. When eight monoclonal antibodies directed to distinct glycan epitopes of plant AGP were screened by enzyme-linked immunosorbent assay (ELISA), three were found to bind to material obtained from lysed cells of *N. punctiforme* and *Nostoc* LBG1 (Fig. 1A). The monoclonal antibodies LM14 (Moller et al. 2008) and MAC207 (Pennell et al. 1989) bound effectively to material from *N.*

punctiforme and showed some preferential occurrence in cell material grown in the presence of nitrate. Recognition of these epitopes in LBG1 was significantly lower, although again showing preference for cells grown in the presence of nitrate (Fig. 1A). By contrast, the LM2 AGP glycan epitope (Smallwood et al. 1996) was not detected in *N. punctiforme* cell material but was present in LBG1 cells and was also more abundant in cells when grown in the presence of nitrate. These results were explored further through immunoblotting of 20-fold concentrated, used growth media from the same two cyanobacterial strains (Fig. 1B). All three monoclonal antibodies bound to some high molecular weight smears >200 kDa, in accordance

Fig. 1. Detection of arabinogalactan protein (AGP) epitopes in free-living *Nostoc* spp. **A**, Enzyme-linked immunosorbent assay analysis of lysed cell suspensions of cyanobacterial strains *Nostoc punctiforme* and *Nostoc* LBG1 with anti-AGP antibodies LM14, MAC207, and LM2. Samples grown in the presence (+) or absence (–) of nitrate and equalized to chlorophyll *a* at $1 \mu\text{g ml}^{-1}$ for coating of microtiter plates. Error bars indicate standard error of the mean of three replicates. **B**, Immunoblots of *N. punctiforme* and *Nostoc* LBG1 growth media from cultures grown with (+) or without (–) a nitrate source with anti-AGP monoclonal antibodies LM14, MAC207, and LM2. In both sets of analyses, LM14 and MAC207 show a preference for *N. punctiforme* cells or exudates and LM2 for LBG1 cells or exudates.

with the properties of plant AGP when run on sodium dodecyl sulfate (SDS) polyacrylamide gels. The differential recognition of media reflected that of lysed cell material observed in ELISA analysis, in that LM14 and MAC207 bound specifically to molecules in the media of *N. punctiforme* and LM2 to material from the media of *Nostoc* LBG1.

These observations indicate that specific epitopes, known to be present in some plant AGP, were present in the cells and growth media of free-living *Nostoc* spp.; that the epitopes were carried by high molecular weight molecules with properties equivalent to those of plant AGP; that there was a differential occurrence of specific epitopes (recognized by MAC207 or LM14 versus LM2) in the two strains of *Nostoc*; and that there was a tendency to detect quantitatively more AGP epitopes in media obtained from *Nostoc* strains grown in the presence of nitrate.

Indirect immunofluorescence microscopy was used to further investigate the occurrence of the LM14, MAC207, and LM2 AGP epitopes in *Nostoc* strains (Fig. 2). Five other AGP-specific antibodies (JIM4 and JIM13 to -16) did not bind to *Nostoc* cells and JIM4 was used as a negative control (Fig. 2). In the case of LM14 and MAC207, antibody binding to filaments of *N. punctiforme* was localized to the cell surfaces of vegetative cells in filaments or the extracellular material known to coat them. Antibody binding was generally most abundant in vegetative cells adjacent to heterocysts but absent from the heterocysts themselves (Fig. 2A and B). The epitope recognized by the LM2 antibody was also detected in *Nostoc* LBG1 at the surface of filaments (Fig. 2C). At the surface of *Nostoc* LBG1 filaments, the LM14 or MAC207 epitopes were only observed at very low levels and were often not observed at all, and the same was true for *N. punctiforme* filaments with LM2 (data not shown). This supports the conclusions of ELISA analyses that AGP glycan epitopes are present in these organisms.

Cultures of *Nostoc* LBG1 were induced to form hormogonia by transferring exponentially growing cultures from media lacking nitrate (but supplemented with 35 mM glucose) into the same medium lacking glucose. The three anti-AGP antibodies identified previously as interacting with the two *Nostoc* spp. were used to detect AGP epitopes at the surface of LBG1 hormogonia (Fig. 3). Only bound LM2 and its epitope was associated with the exopolysaccharide sheath known to coat cyanobacterial filaments (Pereira et al. 2009).

In silico analysis indicates that the *N. punctiforme* genome encodes proteins with the characteristics of AGP core proteins.

Previous studies have outlined a method for identifying AGP protein-coding sequences in *Arabidopsis* (Schultz et al. 2002; Showalter et al. 2010) and in *Triticum aestivum* and *Oryza sativa* (Faik et al. 2006) genomes. It should be noted that these studies were all undertaken in plants and, if AGP core protein sequences are present in cyanobacteria, these may have characteristics very different from those of plants; however, it was considered that the use of these techniques was a starting point from which further analysis could be undertaken. Application of these methods to the *N. punctiforme* genome led to the identification of several proteins that appear to possess some of the unusual characteristics of AGP core proteins; for example, regions rich in PAST (found in PA/PT/PS repeats) and a signal peptide targeting the protein to the cell surface. Schematic outlines of the structures of these putative *Nostoc* AGP core proteins are shown in Figure 4. Regions containing the sequence ([AST]-P-X_(0,10)-[AST]-P), where [AST]-P motifs are separated by less than 11 other amino acids, are good candidates for glycomodules because this sequence may be readily arabi-

noglycosylated (Schultz et al. 2002). All the candidate protein sequences contain these regions (Table 1).

Putative representatives of all three classes of AGP core protein sequences known for plant AGP can be identified in the *N. punctiforme* genome. Of the five candidate putative proteins identified, two (NPUN_F0888 and NPUN_R0902) most closely resemble the classical AGP family, having an N-terminal signal sequence (targeting the protein to the cyanobacterial cell wall), a glycomodule rich in PAST, and no fasciclin domains (Fig. 4) (Schultz et al. 2000). One protein (NPUN_F4498) resembles an AG peptide, being between 50 and 75 amino acids in length and >35% PAST, along with a signal peptide (Fig. 4) (Schultz et al. 2002). The other two proteins (NPUN_R4210 and NPUN_F4094) resemble FLA, in that they contain fasciclin domains along with the characteristic PAST-rich glycomodules and the signal peptide (Fig. 4) (Johnson et al. 2003). It should be noted that, whereas some plant AGP contain GPI anchors, debate continues as to whether GPI anchors have been found in prokaryotes (Chatterjee and Mayor 2001; Chungjatupornchai and Fa-aaroonsawat 2009) and, hence, they may not be expected to be found in the proteins identified here.

The two putative FLA identified by this study contain several recognized domains as identified by InterProScan (Zdobnov and Apweiler 2001). NPUN_F4094 contains a domain with homology to the FAS1 fasciclin domain family. The same region also shows homology to the FimH-like protein family (PANTHER protein subfamily PTHR10900:SF8) involved in cell adhesion. NPUN_R4210 contains regions of the same families identified for NPUN_F4094, along with an S-layer homology region (InterPro family IPR001119), which form layers of glycoproteins known to coat bacterial surfaces (Mesnage et al. 2000). Both putative FLA identified here have been previously identified by proteomic studies of the *N. punctiforme* proteome. NPUN_F4094 (labeled by its accession number, ZP_00111758) was identified as a protein involved in cell motility, secretion, or cell envelope biogenesis in the soluble cell fraction of *N. punctiforme* cells grown in the absence of a nitrate source, demonstrating the presence in vivo of this protein in the proteome (Ran et al. 2007). It should be noted, however, that this attribution of function was based on sequence analysis alone. Similarly, NPUN_R4210 (labeled by its accession number, ZP_00108174) was found to be one of only four surface- or membrane-associated proteins upregulated in *Nostoc* spp. isolated from *Gunnera* glands (Ekman et al. 2006). Again, these were designated as membrane-associated via sequence analysis rather than a direct attribution of function. Both putative FLA contain regions resembling AG glycomodules containing noncontiguous [AST]-P repeats, as well as the distinctive ([AST]-P-X_(0,10)-[AST]-P)_x repeats, as used to identify putative AGP (Table 1) (Faik et al. 2006; Showalter et al. 2010).

The two classical AGP-like proteins identified here also have high numbers of characteristic [AST]-P and ([AST]-P-X_(0,10)-[AST]-P)_x repeats. NPUN_R0902 contains a signal peptide identified by InterProScan, suggesting that the protein is located in the cell membrane. This would be expected in an AGP acting as an extracellular signaling molecule. NPUN_F0888 contains two recognized domains: an XGLTT gated calcium channel region (ProDom family ID PD003992) and a cell wall peptidoglycan anchor (PD153432), as identified by InterProScan (Zdobnov and Apweiler 2001). These domains suggest that the protein is localized to the cell wall or envelope.

The predicted AG peptide, NPUN_F4498, contains a signal peptide region and overlapping transmembrane domain. Schultz and associates (2002) suggest that AG peptides can only be defined if they contain two or more AP/SP motifs in the mature protein. SignalP (Bendtsen et al. 2004) predicts the cleavage of the signal peptide either at position 25-26 or 35-36—this

Fig. 2. A, Indirect immunofluorescence detection of arabinogalactan protein (AGP) glycan epitopes LM14 **B,** MAC207, and **C,** LM2 at the surface of free-living but symbiotically competent cyanobacterial species. Paired images of fluorescein isothiocyanate (FITC) immunofluorescence and bright field (upper) with immunofluorescence alone (lower). LM14 and MAC207 are detected at the surface of *Nostoc punctiforme* and LM2 at the surface of *Nostoc* LBG1 filaments. **D,** Nonbinding antibody JIM4 is shown as a negative control. H = heterocyst. Scale bars = 10 μm.

cleavage site may be vital, because cleavage at the former site generates a peptide with one [AST]-P-X_(0,10)-[AST]-P repeat (composed of an APAP sequence) and one TP repeat, whereas cleavage at the latter site results in a peptide with one AP and one TP repeat. Hence, depending on which cleavage site is found in the protein *in vivo*, this may or may not be a true AG peptide candidate.

The LM14, MAC207, and LM2 AGP glycan epitopes are detected in gland mucilage and at the cyanobacterial-plant interface in the *Gunnera*–*Nostoc* symbiosis.

Immunofluorescence analysis of thick sections of outer gland regions of uninfected *Gunnera manicata* with covering mucilage indicated the presence of the LM14, MAC207, and LM2 AGP epitopes. Micrographs for LM14 and LM2 are shown in Figure 5A and B. Sections of naturally infected stem glands in regions of intracellular symbionts also indicated the presence of the same three epitopes. The LM14 epitope was detected in the plant cell walls surrounding the symbiotic colonies and also at the surface of the symbiotic colonies themselves (Fig. 5C). This was also the case for MAC207 (not shown). By contrast, the LM2 epitope had a different profile of occurrence, being abundantly detected in the symbiotic colonies of *Nostoc* spp. within the *G. manicata* gland but was not detected in the plant cell walls adjacent to the symbiotic colonies (Fig. 5D).

The LM2 AGP glycan epitope is temporally regulated during the formation of the *Nostoc*–*Blasia* symbiosis.

The *Nostoc*–*Blasia* symbiosis can be readily established in uninfected plants (Adams and Duggan 2011). A 72-h time-course experiment was carried out to assess the expression of AGP epitopes during the establishment of the *Blasia*–*Nostoc*

symbiosis. LM14, MAC207, and LM2 were used to probe the *B. pusilla* symbiosis during the infection process. The auricles of uninfected *B. pusilla* and *B. pusilla* and *Nostoc* cells did not show any binding of LM14 or MAC207 antibodies throughout an infection time course of 72 h (data not shown). However, the LM2 AGP glycan epitope was specifically detected in auricle structures when *B. pusilla* was grown in sterile culture, prior to contact with cyanobacterial filaments, whereas no epitope was detected in nonsymbiotic thallus material (Fig. 6A, 0 h, marked with a “T”). As the infection time course proceeded, the auricles began to lose their reactivity with the LM2 antibody. By 48 h, hormogonia had started to invade the symbiotic space between the auricle wall and the inner papilla, encircling the inner papilla itself (Fig. 6B; hormogonia may be seen by red autofluorescence). The LM2 epitope remained detectable on the outer papillae but not on the inner. After 60 h, many more cyanobacterial cells were present in the auricles and the LM2 epitope was no longer detected on the papillae (Fig. 6C). After 72 h, the auricles were tightly packed with cyanobacterial cells. The ends of some filaments could be seen protruding from the auricle, and these reacted strongly with LM2 (Fig. 6D). As with the 60-h time point, the LM2 epitope was not detected on either the inner or outer papillae.

DISCUSSION

Putative AGP are present in free-living cyanobacteria.

Preliminary work had indicated that some anti-AGP antibodies may bind to concentrated *Nostoc* growth media (Snogerup 1997). The results presented here using ELISA, immunoblotting, and immunofluorescence procedures demonstrate that symbiotically competent *Nostoc* spp. express a range of carbohydrate epitopes thought previously to be exclusive to plant AGP. The epitopes of monoclonal antibodies LM14,

Fig. 3. A, Indirect immunofluorescence detection of the LM2 epitope at the surface of *Nostoc* LBG1 hormogonia. **B,** In this case, MAC207 does not bind. Cultures were 50 h postinduction. Paired images of immunofluorescence and bright field (upper) and immunofluorescence only (lower). Arrows indicate the edge of the mucilage sheath detected by the LM2 antibody. Scale bar = 20 μ m.

MAC207, and LM2 all contain glucuronic acid (Moller et al. 2008; Smallwood et al. 1996; Yates et al. 1996; *unpublished observation*) that is known to decorate core AG structures. The similarity in the binding profiles of LM14 and MAC207 in the diverse assays may indicate that they recognize structurally related epitopes. These observations suggest that AGP may be present in long-term laboratory-cultured, free-living *Nostoc* strains, such as *N. punctiforme* and *Nostoc* LBG1, which were originally isolated from plant symbioses but have been cultured away from plants for more than 20 years.

The occurrence of the AGP glycan epitopes, as detected by immunofluorescence, at the surface of cyanobacterial filaments was not uniform and the LM14 or MAC207 epitopes were generally most abundant at the surface of cells adjacent to heterocysts but appeared to be absent from the heterocysts them-

selves (Fig. 2). Heterocyst cell walls are thicker and more complex than those of vegetative cells, having a polysaccharide layer, a glycolipid layer, and an outer fibrous layer external to the normal cell envelope (Adams and Duggan 1999). How the pattern of AGP glycan epitope occurrence relates to the filament cell heterogeneity or vegetative cell or heterocyst function is not clear at this stage.

Although previous studies of AGP core proteins have shown them to be highly variable in sequence and secondary structure (Faik et al. 2006; Schultz et al. 2000, 2002), we present further evidence that *Nostoc* cultures may express AGP by the identification of five putative AGP core proteins in the *N. punctiforme* translated proteome. All five putative core proteins possess regions strongly resembling AG glycomodules, and all are secreted proteins, as would be expected for AGP.

Fig. 4. Schematic diagram to show candidate arabinogalactan proteins encoded in the *Nostoc punctiforme* genome. Included with the *Nostoc* database numbers is the complete protein length in amino acids. Lengths are relatively scaled. Putative protein domains are indicated.

Table 1. Summary of glycomodule motifs in putative arabinogalactan proteins (AGP) of *Nostoc punctiforme*

Gene locus (GenBank)	Protein description	Protein (aa) ^b	Number of glycomodule motifs ^a			
			[AST]-P	([AST]-P-X _(0,10) -[AST]-P)	[AST]-P-P	[AST]-P-P-P
Classical AGP						
NPUN_F0888	Putative uncharacterized protein	233	3	15	4	0
NPUN_R0902	Putative uncharacterized protein	482	2	14	0	0
AG-peptides						
NPUN_F4498	Putative uncharacterized protein	63	1 (2) ^c	1 (0) ^c	0	0
Fasciclin-linked AGP						
NPUN_R4210	β-Ig-H3/fasciclin	563	3	2	0	0
NPUN_F4094	β-Ig-H3/fasciclin	276	3	1	1	0

^a These glycomodule motifs were used by Faik and associates (2006) as characteristic of AGP. Letters enclosed in square brackets indicate the choice of amino acid residues at this position in order to designate a glycomodule motif.

^b Protein size = number of amino acids (aa).

^c Two counts are provided due to difference in prediction of signal peptide cleavage site by SignalP program.

Fig. 5. Indirect immunofluorescence detection of arabinogalactan protein (AGP) epitopes in *Gunnera manicata* stem glands at the symbiotic surface of *Nostoc*–*Gunnera* symbioses. Paired images of fluorescein isothiocyanate (FITC) immunofluorescence and bright field (upper) with immunofluorescence alone (lower) are presented for uninfected gland surfaces with mucilage are shown for **A**, LM14 and **B**, LM2. Paired images of FITC immunofluorescence and red autofluorescence indicating presence of cyanobacteria (upper) with immunofluorescence alone (lower) are shown for sections through regions with intracellular symbionts for **C**, LM14 and **D**, LM2. Arrows indicate AGP detection in mucilage at gland surfaces. GM = gland mucilage, GC = gland cells, and PCW = plant cell walls adjacent to cells with intracellular symbionts (ICs). Scale bars = 20 μm.

Post-translational glycosylation of AGP peptide cores is required to generate the large-branched glycan structure of AGP but evidence of glycosylation in cyanobacteria is somewhat sparse. SwmA, a protein implicated in motility in *Synechococcus* sp. (a nonflagellated marine cyanobacterial species), has been shown to be glycosylated and to contain N-glycosylation sites (Brahamsa 1996). Oscillin is another motility-associated protein found on the cell surface and which has a glycosylated structure, containing xylose, glucose, rhamnose, fucose, arabinose, and galactose (Hoiczky and Baumeister 1997). More recently, a glycoprotein has been identified in the toxic cyanobacterium *Microcystis aeruginosa* (Zilliges et al. 2008). This protein is implicated in cell-to-cell contact and is post-translationally modified by the addition of O-linked glycans. Hence, although little work has been done in the field, it appears that cyanobacteria possess the ability for post-translational glycosylation and these modified proteins are involved in cell surface processes.

Although we have demonstrated the occurrence of AGP glycan epitopes and identified putative AGP core protein sequences, we have not made a link between expressed (glyco)proteins and the specific epitopes. This is a challenging area, and links between glycan epitopes and specific AGP core protein sequences are not yet easy to establish for plant AGP (Ellis et al. 2010; Seifert and Roberts 2007).

AGP epitopes at symbiotic interfaces.

The role of putative AGP at the surface of free-living cyanobacteria grown for thousands of generations in laboratory con-

ditions is unknown. However, the observation that the same set of three AGP epitopes is detected in the mucilage of *G. manicata* glands raises the possibility that they may play a role in the initiation or maintenance of symbioses—although this remains to be demonstrated. *Gunnera* gland mucilage has previously been implicated in the infection process (Rasmussen et al. 1994, 1996; Towata 1985). It is of interest that the LM14- or MAC207-reactive epitopes were also present in the surrounding cell walls but that the LM2-reactive epitope was specific to the symbiotic colonies within the gland structure, suggesting that the epitope may be produced by the symbiont.

NPUN_R4210, identified in this study as a putative FLA, is upregulated in symbiotically associated *Nostoc* spp., suggesting that it has a role in the maintenance of a stable symbiosis (Ekman et al. 2006). Another fasciclin-containing protein, NPUN_F4094, also identified here as being a putative FLA, has been detected in a functional proteomics study as a protein involved in cell motility, secretion, or cell envelope biogenesis (Ran et al. 2007). Fasciclin domain proteins have previously been implicated in cyanobacterial symbioses (Ekman et al. 2006; Paulsrud and Lindblad 2002).

AGP have previously been shown to be present in the cell walls of liverworts, although those tested do not host cyanobionts (Basile and Basile 1987). In axenic (uninfected) *Blasia* cultures, the LM2 AGP glycan epitope was specifically detected on the inner wall of the auricle as well as the inner and outer papillae but not in the surrounding nonsymbiotic thallus cells (Fig. 6, 0 h). As the auricle became populated with cyanobacterial hormogonia (also expressing the LM2 epitope),

Fig. 6. Indirect immunofluorescence detection of the LM2 arabinogalactan protein (AGP) epitope in *Blasia* symbiotic structures (auricles) through a 72-h infection time course. Paired images of (top) bright field, cyanobacterial autofluorescence (red), and immunofluorescence (green) with (bottom) autofluorescence/immunofluorescence only for **A**, 0 h; **B**, 48 h; **C**, 60 h; and **D**, 72 h. Inner and outer papillae (IP and OP, respectively) are indicated along with nonsymbiotic thallus material (T). Scale bar = 20 μ m.

LM2 epitope detection on both inner and outer papillae was reduced. By 60 h, the LM2 epitope was no longer detected on the outer papilla but abundant on adjacent hormogonia (Fig. 6, 60 and 70 h). In the case of the outer papillae, this seems to be due to loss of the epitope, because these papillae always remain accessible to the antibody but show significant reduction in LM2 binding (Fig. 6, 60 and 72 h). However, as the cyanobacteria completely fill the interior of the auricle, the lack of LM2 epitope detection at the interior auricle wall and inner papillae may be due to restricted access. This appears to be supported by analysis at 60 and 72 h, where the ends of cyanobacterial filaments protruding from the auricle are bound by the antibody, whereas those parts of the filament within the auricle are not. Signaling and attraction between hormogonia and the auricle structure is known to occur (Nilsson et al. 2006).

Gunnera stem gland mucilage is known to contain high levels of sugars such as arabinose (Khamar et al. 2010), a possible hormogonia attractant which may be released from polymers such as AGP by the extracellular enzyme arabinofuranosidase, the expression level of which is elevated in *G. manicata* stem tissue containing glands compared with that lacking glands (Khamar et al. 2010). There are parallels between *Gunnera* gland development with adventitious root formation, and it has been suggested that glands may be modified adventitious roots (Chiu et al. 2005). Plant AGP have been extensively implicated in root generation and growth (Knox et al. 1991; Seifert and Roberts 2007; van Hengel and Roberts 2003).

Did plant AGP originate in cyanobacteria?

Evidence is growing that a large amount of genetic information in plants originated from the cyanobacterial ancestors of plastids, which first appeared 1.2 billion years ago when a cyanobacterium invaded a biciliate protozoan (Cavalier-Smith 2000; Deusch et al. 2008). A study of gene acquisition from cyanobacteria in four eukaryotes has estimated that 14% of plant proteins are of cyanobacterial origin and that, of all cyanobacterial genomes, that of the genus *Nostoc* is the closest to that of plants (Deusch et al. 2008). This analysis implies that the ancestor of plastids was an organism similar to filamentous, heterocyst-forming cyanobacteria typified by *Nostoc* spp. (Deusch et al. 2008). Therefore, it is possible that AGP proteoglycans may represent a class of glycosylated proteins that originally evolved in cyanobacteria, and that have been transferred to a plant host at some point in the last 500 million years. It has also been speculated that the capacity for cellulose synthesis is cyanobacterial in origin (Nobles and Brown 2004; Nobles et al. 2001). It is of interest in this regard that AGP are implicated in several aspects of cell wall function in plants (Ellis et al. 2010; Seifert and Roberts 2007) and it is possible that several aspects of plant cell wall structure and assembly originated in cyanobacteria.

In summary, we present evidence that free-living cyanobacteria of the genus *Nostoc* produce AGP glycan epitopes and contain sequences of putative AGP core proteins. The same specific glycan epitopes are present in the symbiotic structures of both *G. manicata* and *B. pusilla*. Although we are, at present, unable to show a direct relationship between the specific AGP glycan epitopes identified by immunochemistry and the predicted AGP core proteins, we suggest that, taken together, the results imply that *Nostoc* spp. can express AGP-like proteins, previously thought exclusive to the plant kingdom.

MATERIALS AND METHODS

Culture of cyanobacterial strains.

N. punctiforme ATCC 29133 and *Nostoc* LBG1 (a symbiotic strain isolated from a hornwort) were grown using variations

of liquid BG11 medium (Rippka et al. 1979), either with (+N) or without (–N) added sodium nitrate (17 mM). In addition to these standard nutrients, glucose was included in *Nostoc* LBG1 –N cultures at a final concentration of 35 mM to suppress the natural tendency of this cyanobacterium to transform into hormogonia when grown in nitrogen-fixing culture. All cultures were incubated at 100 rpm at 30°C under continuous light of 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR). To generate hormogonia, a stable culture of *Nostoc* LBG1 grown in BG11-N supplemented with 35 mM glucose (as described above) was centrifuged ($3,500 \times g$, 2 min) and washed twice with BG11-N before being transferred to BG11-N with no added glucose. This culture was incubated overnight under standard conditions (described above) and hormogonia differentiation was checked by microscopy to confirm that the proportion of hormogonia to vegetative filaments was >90%.

Plant materials.

Samples of naturally infected *G. manicata* ‘Linden’ glands were dissected from plants grown at Harewood House Estate, West Yorkshire, United Kingdom. Uninfected samples of the liverwort *B. pusilla* L. were obtained from Paula Duggan (University of Leeds) and aseptically subcultured into BG11+N media in conical flasks every month. These were kept at 22°C on an orbital shaker (100 rpm) under artificial light at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PAR and a cycle of 12 h of light and 12 h of darkness.

AGP-directed monoclonal antibodies.

The eight rat anti-AGP monoclonal antibodies used in this study were MAC207 (Pennell et al. 1989), JIM4 (Knox et al. 1989), JIM13, JIM14, JIM15, JIM16 (Knox et al. 1991), LM2 (Smallwood et al. 1996), and LM14 (Moller et al. 2008). LM15, an anti-xyloglucan monoclonal antibody, was used as a negative control for ELISA (Marcus et al. 2008). In all cases, the antibodies were used as unpurified hybridoma cell culture supernatants.

ELISA analysis of AGP in cyanobacterial cell material.

The chlorophyll a (chl a) content of cyanobacterial cultures was determined by a methanol extraction assay and the absorbance read at 665 nm (Tandeau de Marsac and Houmard 1988; although the extinction coefficient used was modified to 12.9447 as described by Ritchie 2006). Cultures were then diluted to a chl a value of 1 $\mu\text{g/ml}$ to standardize loading for the immunoassay and used to coat the ELISA microtiter plate (Immuno 96 MicroWell plates; NUNC, Langenselbold, Germany) overnight at 4°C. The plates were dried and stored at –20°C before use. ELISA plates were blocked with 3% (wt/vol) milk protein in phosphate-buffered saline (MP/PBS) overnight, 200 μl per well, before washing nine times in tap water. Primary rat antibodies diluted 10-fold in 3% MP/PBS were added and incubated for 1 h at room temperature, before washing (nine times in tap water). Plates were then incubated with a 1,000-fold dilution of secondary antibody (anti-rat immunoglobulin G [IgG] [whole molecule] coupled to horseradish peroxidase [HRP]) (Sigma-Aldrich, Gillingham, U.K.) in MP/PBS for 1 h at room temperature, before being washed (nine times in tap water). The HRP substrate (18 ml of H_2O , 2 ml of 1 M sodium acetate buffer [pH 6.0], 200 μl of 3,3',5',5'-tetramethyl benzidine [Sigma T-2885, 10 mg/ml in dimethyl sulfoxide], and 20 μl of 6% [vol/vol] H_2O_2) was then added and allowed to develop for 5 min before 2.5 M sulfuric acid (30 $\mu\text{l/well}$) was added to stop the reaction. Absorbance values were read at 450 nm using a Bio-tek Instruments ELX800 automated universal microplate reader. Rat antibody LM15 was used as a negative control for these analyses and absorbance

values for the nonspecific binding of this antibody were subtracted from the values determined with anti-AGP antibodies.

Immunoblotting of growth media.

Growth medium (50 ml) was taken from 10-day-old cultures of *N. punctiforme* and *Nostoc* LBG1 and filtered through a 0.45- μ m filter to remove cells. These media were then freeze dried and resuspended in sterile distilled water to a 10- to 20-fold increased concentration. Samples were then separated using standard SDS polyacrylamide gel electrophoreses with 30 μ l added per lane. An 8- to 220-kDa molecular weight marker set (ColorBurst; Sigma-Aldrich) was included on each gel. The resulting blots were then blocked overnight (4°C) in 3% MP/PBS. The blots were then washed thoroughly with water and rat monoclonal antibodies LM14, MAC207, and LM2 were diluted 10-fold in 3% MP/PBS for 1 h at room temperature. After 5-min washes three times with PBS, the blots were incubated with the secondary antibody (anti-rat IgG coupled to HRP [Sigma-Aldrich] diluted 1,000-fold in 3% MP/PBS for 1.5 h at room temperature) and the PBS washing steps were repeated. The blots were then developed using HRP color substrate (25 ml of H₂O, 5 ml of MeOH containing 10 mg/ml 4-chloro-1-naphthol, and 30 μ l of 6% [vol/vol] H₂O₂) before being washed in water.

Immunofluorescence microscopy.

Cyanobacterial cells were fixed O/N in a 4% (wt/vol) formaldehyde (as for plant material, discussed below), aliquotted onto Vectabond-coated multiwell slides (Vector Laboratories, Peterborough, U.K.) and allowed to dry at 37°C for 1 h before being blocked for 30 min with 5% MP/PBS. Wells were then incubated with primary rat antibodies diluted 10-fold in 5% MP/PBS for 1 h at room temperature. A negative control well was incubated with 5% MP/PBS only. After washing with PBS (three times, 5 min), slides were incubated with the secondary antibody, anti-rat IgG coupled to fluorescein isothiocyanate (Sigma) diluted 100-fold in 5% MP/PBS for 1.5 h at room temperature in darkness. After washing (5 min, three times, with PBS), slides were mounted using Citifluor AF2 antifade reagent (glycerol suspension; Agar Scientific Stansted, U.K.). Excised samples of environmentally infected *G. manicata* glands (from Harewood House Estate, West Yorkshire, U.K.) were fixed, wax-embedded, and sectioned to a thickness of 10 μ m as described (Marcus et al. 2008). Sections were analyzed by indirect immunofluorescence with anti-AGP monoclonal antibodies using the same protocol as described for cyanobacterial samples above. All samples were examined using an Olympus BX-61 microscope with epifluorescence irradiation. Images were captured using a Hamamatsu ORCA285 camera and Volocity software.

Bioinformatics.

The *N. punctiforme* proteome was obtained from the Swiss-Prot/TrEMBL ExPASy proteomics server. Prior studies were used to identify characteristic sequences (Faik et al. 2006; Schultz et al. 2000, 2002).

Classical AGP. Proteins rich in PAST in the *Nostoc* proteome were identified using the PERL script as described (Schultz et al. 2002), set to isolate only those proteins with >50% PAST. Resulting proteins were then analyzed using SignalP (Bendtsen et al. 2004) to identify those with secretion signals and the location of secretion signal cleavage sites, and InterProScan (Zdobnov and Apweiler 2001) to identify any conserved domains. Sequences with secretion signals were further analyzed by eye using the Jalview program (Waterhouse et al. 2009) to identify potential AG glycomodules.

AG peptides. Using the PERL script (Schultz et al. 2002), all proteins shorter than 75 residues were identified, and then ana-

lyzed to isolate those possessing >35% PAST. These were then analyzed as for classical AGP (discussed above).

FLA. All cyanobacterial fasciclin-containing proteins were identified from the InterPro IPR000782 FAS1 domain family. A consensus sequence was then generated using the MUSCLE multiple sequence alignment system (Edgar 2004a and b) and Jalview (Waterhouse et al. 2009). This sequence then identified proteins containing fasciclin domains in the *N. punctiforme* proteome using BLASTp ($E = 0.001$, BLOSUM62 matrix) (Altschul et al. 1997). These proteins were then analyzed using the PERL script (Schultz et al. 2002) to isolate those containing >35% PAST. These sequences were then analyzed as for classical AGP (see above).

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