Multiple Roles of Soluble Sugars in the Establishment of *Gunnera-Nostoc* Endosymbiosis[1][OA]

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Gunnera plants have the unique ability to form endosymbioses with N_2-fixing cyanobacteria, primarily *Nostoc*. Cyanobacteria enter *Gunnera* through transiently active mucilage-secreting glands on stems. We took advantage of the nitrogen (N)-limitation-induced gland development in *Gunnera manicata* to identify factors that may enable plant tissue to attract and maintain cyanobacteria colonies. Cortical cells in stems of N-stressed *Gunnera* plants were found to accumulate a copious amount of starch, while starch in the neighboring mature glands was nearly undetectable. Instead, mature glands accumulated millimolar concentrations of glucose (Glc) and fructose (Fru). Successful colonization by *Nostoc* drastically reduced sugar accumulation in the surrounding tissue. Consistent with the abundance of Glc and Fru in the gland prior to *Nostoc* colonization, genes encoding key enzymes for sucrose and starch hydrolysis (e.g. cell wall invertase, α-amylase, and starch phosphorylase) were expressed at higher levels in stem segments with glands than those without. In contrast, soluble sugars were barely detectable in mucilage freshly secreted from glands. Different sugars affected *Nostoc*'s ability to differentiate motile hormogonia in a manner consistent with their locations. Galactose and arabinose, the predominant constituents of polysaccharides in the mucilage, had little or no inhibitory effect on hormogonia differentiation. On the other hand, soluble sugars that accumulated in gland tissue, namely sucrose, Glc, and Fru, inhibited hormogonia differentiation and enhanced vegetative growth. Results from this study suggest that, in an N-limited environment, mature *Gunnera* stem glands may employ different soluble sugars to attract *Nostoc* and, once the cyanobacteria are internalized, to maintain them in the N_2-fixing vegetative state.

Nitrogen (N) is an essential element for plant growth, but availability of reduced N in the soil is often limiting. Representatives from a wide range of land plants have evolved the ability to form associations with N_2-fixing microbes (Franche et al., 2009). Associations between rhizobia and legume plants are well-characterized examples of plant-bacterial N_2-fixing symbioses. Unlike rhizobia, which generally exhibit narrow host ranges (Kistner and Parniske, 2002), N_2-fixing cyanobacteria are able to form productive associations with a broad range of plants, including bryophytes (hornworts and liverworts), ferns (*Azolla*), gymnosperms (cycads), and angiosperms (*Gunnera*; for review, see Rai et al., 2000; Adams et al., 2006). Free-living cyanobacteria within the genus *Nostoc* can fix N in specialized microoxic cells called heterocysts. The ability of *Nostoc* to fix N independent of a host environment may facilitate the formation of symbioses with a wide range of plants. Understanding the physiological conditions that enable a plant host to enter into symbiotic associations with cyanobacteria may allow us to extend the benefit of biological N fixation to crops outside the legume family.

Nostoc has the ability to differentiate not only into filaments bearing heterocysts but also into transiently motile filaments, known as hormogonia, which enable the cyanobacteria to enter plants (Meeks and Elhai, 2002). *Nostoc* can be induced to form hormogonia by different environmental stimuli and by a hormogonia-inducing factor released from N-stressed host plants (Meeks and Elhai, 2002; Adams et al., 2006). The attraction of hormogonia to plants is much less specific than that of rhizobia. Hormogonia are attracted to root extracts from either host or nonhost plants and even to certain simple sugars, such as Ara, Glc, and Gal (Nilsson et al., 2006). After entering a plant host, hormogonia revert back to filaments with N_2-fixing heterocysts. Inside the host, further hormogonia formation is suppressed, and heterocysts appear at a frequency of about 30% to 40%, 3- to 4-times higher than that found in free-living *Nostoc* (Meeks and Elhai, 2002). Although free-living *Nostoc* species can support N_2 fixation through photosynthesis, under symbiotic conditions they rely on photosynthate from the host plant. In general, the sugars (Suc, Glc, and Fru) known to support heterotrophic growth in the dark by free-living cyanobacteria coincide with those that support nitrogenase activity in *Nostoc*-plant associations (Meeks and Elhai, 2002). However, the *Nostoc-Gunnera* association may be exceptional; only Glc and Fru have been shown to sustain nitrogenase activities (Man and

Silvester, 1994; Wouters et al., 2000), although Suc and dextrin were able to keep *Nostoc* alive without light (Wouters et al., 2000). It is evident from cyanobacterial studies that the plant hosts have evolved the ability to regulate cyanobacterial growth and differentiation during symbiotic associations (Meeks and Elhai, 2002). However, because most studies of plant-cyanobacterial associations have focused on the cyanobacterial partner (e.g. Wang et al., 2004; Ekman et al., 2006), the mechanisms through which plant hosts attract, internalize, and maintain cyanobacteria remain to be elucidated (Adams et al., 2006).

The *Nostoc-Gunnera* association is an ideal system with which to study plant-cyanobacteria symbioses, not only because *Gunnera* is the only genus of angiosperms known to form endosymbioses with N_2-fixing cyanobacteria but also because the association between the two can be readily established in the laboratory (Bergman et al., 1992; Chiu et al., 2005). *Nostoc* hormogonia enter *Gunnera* plants through specialized glands located on the stem. As the gland matures, it secretes polysaccharide-rich mucilage that attracts cyanobacteria (Nilsson et al., 2006), supports their growth on the gland surface (Towata, 1985; Chiu et al., 2005), and permits further hormogonia differentiation (Rasmussen et al., 1994). From there, hormogonia enter the gland and penetrate cells near the base of the gland in the stem (Bonnett, 1990; Bergman et al., 1992). Although each gland is only transiently capable of accepting cyanobacteria, new glands continue to form on the stem at the base of each new leaf.

In contrast to the development of nodules in legumes, which requires a complex exchange of signals between the two symbiotic partners (Cooper, 2007), stem gland development in *Gunnera* takes place in the absence of cyanobacteria (Bonnett, 1990). N limitation, however, is a prerequisite for stem gland development (Chiu et al., 2005), as it is for nodulation (Barbulova et al., 2007). We have taken advantage of the N-deficiency-induced gland development in *G. manicata* to identify factors that enable *Gunnera* to form endosymbiosis with cyanobacteria. This study investigated changes in the carbohydrate metabolism during *Gunnera* gland development and discovered that tissue in the mature glands accumulated high levels of soluble sugars prior to the arrival of cyanobacteria. In agreement with this finding, several key genes encoding enzymes for starch/Suc hydrolysis were expressed at higher levels in the gland compared to the stem. Furthermore, we found that various sugars cyanobacteria may encounter as they approach *Gunnera* glands as opposed to those they would encounter within plant cells differentially affected *Nostoc*'s ability to form motile hormogonia.

RESULTS

Accumulation of Starch in Stems of N-Deprived *G. manicata* Seedlings

Glands on hypocotyls of *G. manicata* seedlings did not become mature when they were maintained on N-replete medium (Fig. 1A), consistent with a previous report (Chiu et al., 2005). In contrast, mature glands developed normally in plants grown on N-limited medium (Fig. 1, C and E). Internal structures of developing glands were analyzed by making cross sections through glands on hypocotyls. Staining fresh stem sections with KI/I_2 revealed numerous starch-filled amyloplasts (black dots in Fig. 1, D and F) in the cortex of N-deprived seedlings but not in seedlings from N-replete medium (Fig. 1B). Although amyloplasts could also be found in cells within the mature glands on N-deprived seedlings, they were either less abundant (Fig. 1D) or greatly reduced in size (Fig. 1F) compared to those present in the cortex of the stem.

Accumulation of Reducing Sugars in Stems of N-Deprived *G. manicata* Seedlings

The absence of starch granules in mature glands led us to wonder whether starch in the glands may be hydrolyzed to soluble sugars during gland maturation. To test this hypothesis, Benedict's test for reducing sugars was performed on stem segments from *Gunnera* seedlings grown on sugar-free medium with (N+) or without (N−) combined N. Reducing sugars convert Cu^{2+} to Cu^+, changing the blue color of
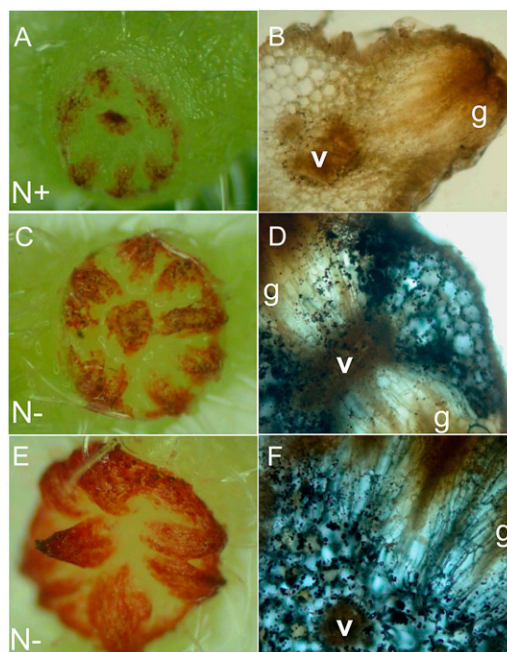

Figure 1. Starch accumulation patterns in hypocotyls of *G. manicata* seedlings. A, An immature gland on the hypocotyl of a seedling grown on N-replete (N+) medium. B, A cross section through an immature gland as shown in A stained with KI/I_2. C and E, Mature glands marked with anthocyanins on hypocotyls of seedlings from N-limited (N−) medium. D and F, Cross section of the stem through glands shown in C and E stained with KI/I_2. Gland tissue is indicated with a g and vascular tissue at the center of the stem is indicated with a v. Note that starch-stained amyloplasts are mainly in the cortical cells but not in the gland.

Benedict's solution to brick red cuprous oxide precipitate. Sugars that react with Benedict's solution include Glc, Fru, Gal, Ara, and maltose. Homogenates of stem segments (without visible glands) or hypocotyls from seedlings grown on N+ medium retained most of the blue color of Benedict's reagent (Fig. 2A, top row), indicating that there was little reducing sugars in these tissues. However, homogenates of stem segments from plants grown on N-free medium turned the Benedict's reagent from blue to brown (Fig. 2A, bottom row), with the amount of brownish precipitate increasing as the number of glands increased. These results suggest that N-stressed *Gunnera* seedlings accumulate high levels of reducing sugars in the stem, especially in stems with glands.

The connection between gland development and the level of reducing sugars was assessed by comparing stem segments with and without glands from the same individual N-stressed soil-grown seedling. The estimated concentration of total reducing sugars in each sample varied greatly among the plants, with the 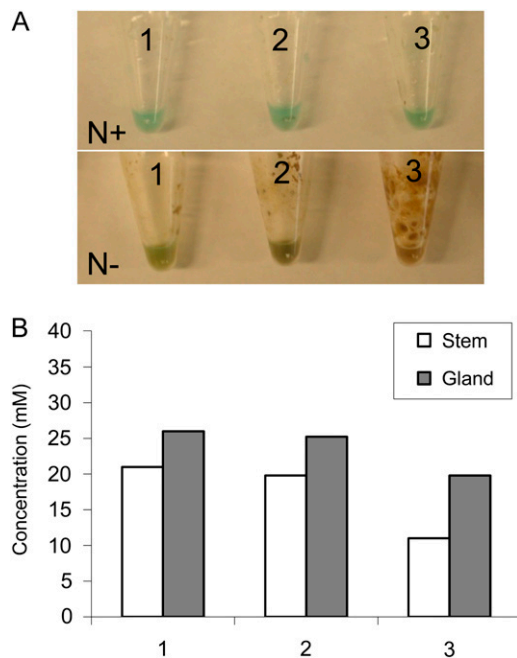smallest (no. 3) of the three plants containing the lowest amount of sugar (Fig. 2B). However, within the same plant, stem segments with glands had a consistently higher level of sugar than segments without glands (Fig. 2B). By comparing the A_{700} reading of each sample to a standard curve obtained using known concentrations of Glc, the concentration of total reducing sugars in stem segments without mature glands was estimated to range between 10 and 20 mM, and the concentration estimated in stem with mature glands was between 20 and 25 mM (Fig. 2B).

Accumulation of Monosaccharides in Tissue of Mature Glands

The identity and concentration of sugars in mature glands were elucidated by extracting soluble sugars from stem segments of N-deprived, soil-grown *G. manicata* seedlings (about 2 months old) possessing mature glands actively secreting mucilage. Quantitative analysis of Glc, Fru, and Suc was performed using the enzymatic NADP-reducing method (Bergmeyer and Bernt, 1974). As shown in Fig. 3A, around 4 nmol mg^{-1} fresh weight (4 mM) of Glc and between 2 to 3 nmol mg^{-1} fresh weight (2−3 mM) of Fru were detected in the three plants analyzed. Although Suc was detected in the samples, it was at a lower concentration (<1 nmol mg^{-1} fresh weight) than that of Glc or Fru. The combined concentration of Glc and Fru in these samples was around 7 mM, which is much lower than the concentration of total reducing sugars (20−25 mM) in the glands estimated using Benedict's test. Due to the specificities of enzymes used in the NADP-reducing method, other reducing sugars in the sample could not be detected.

Soluble Sugar Accumulation in *Nostoc*-Colonized Plants

The effect of *Nostoc* colonization on sugar accumulation in the plant was analyzed by extracting soluble sugars from stem segments of soil-grown, N-deprived *G. manicata* seedlings (taken from the same batch as the ones used for Fig. 3A) about 1 month after *Nostoc punctiforme* inoculation. At the time of harvest, colonization of *Nostoc* on these plants was determined to be successful as judged by the greening of leaves. Stem sections revealed the presence of dark-green *Nostoc* colonies in the upper, younger half of the stems near the shoot tip but not in stems from the lower, older part of the plants. This was most likely due to the fact that glands on the lower part of the stem were no longer functioning at the time of *Nostoc* inoculation. As shown in Figure 3B, for all three plants analyzed, the concentrations of Glc and Fru in the uncolonized older stems were at least 4 times higher than those found in the *Nostoc*-colonized, younger stems from the same plant. The combined concentrations of Glc and Fru in the older stems ranged from 10 to 18 mM, which are much higher than the 7 mM obtained from stems of younger plants before they were inoculated with *Nostoc* (Fig. 3A). These results indicate that, under

Figure 2. Comparison of reducing sugars in *G. manicata* seedlings using Benedict's reagent. A, Top row, homogenates of stems (1 and 2) or a hypocotyl (3) from three different seedlings grown on N-replete (N+) medium. Bottom row, homogenates of stems with no gland (1) or with a single gland (2) or a hypocotyl with three glands (3) from seedlings grown on N-free (N−) medium. B, Estimated concentration of reducing sugars in glands versus stems of three soil-grown, N-stressed seedlings. Stems taken from each plant were divided into segments containing glands (Gland) or those lacking glands (Stem). After completion of the Benedict's reaction, the A_{700} was measured for each sample. The concentration of reducing sugars in each sample was estimated according to a standard curve constructed using solutions with known concentrations of Glc.
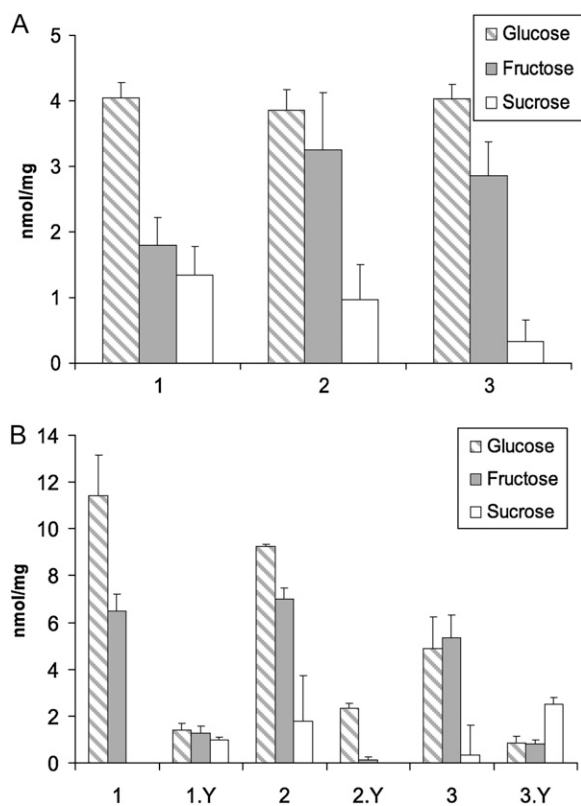
Figure 3. Quantitative analysis of soluble sugars in stem segments containing mature glands from *G. manicata* seedlings. A, Concentrations of soluble sugars in three N-deprived, *Nostoc*-free soil-grown plants. B, Concentrations of soluble sugars in the stems of three *Nostoc*-colonized, soil-grown plants. Each pair (e.g. 1 and 1.Y) represents stem segments from one plant. Segments 1, 2, and 3 are from older, *Nostoc*-free portions of the stem. Segments 1.Y, 2.Y, and 3.Y are from younger, colonized portions of the stem. Note the difference in scale between A and B.

N-limited condition, sugar accumulation in the stem increased as the plant grew older. Furthermore, successful colonization by *Nostoc*, which resupplies the plant with fixed N, greatly reduced measureable free Glc and Fru in the surrounding stem tissue.

Detection of Soluble Sugars in the Mucilage

Mucilage from *Gunnera* glands contains polymers of Ara, Gal, and GlcUAs (Rasmussen et al., 1996). However, no information regarding soluble sugars in the mucilage has been reported. Soluble sugars in the mucilage were characterized using samples collected from the same *Nostoc*-free, soil-grown *G. manicata* plants used for the analysis of soluble sugars in gland tissue (Fig. 3A). In sharp contrast to the high sugar levels within the gland, very low levels of Glc (0.03–0.04 nmol mg^{-1} fresh weight), Fru (0.01–0.02 nmol mg^{-1} fresh weight), and Suc (0.01 nmol mg^{-1} fresh weight) were detected in these mucilage samples. Overall, the concentration of sugars in the muci-

lage amounts to only about one-hundredth of that found in the mature glands from which the mucilage samples were collected (Fig. 3A).

Benedict's test was also used to detect total reducing sugars present in mucilage collected from different batches of soil-grown, N-deprived *G. manicata* seedlings. In agreement with the low level of sugars detected in the above-mentioned analysis, reducing sugar levels in the mucilage collected from seedlings were insufficient to change the color of Benedict's reagent (data not shown).

To see if older plants produce mucilage with higher sugar content, mucilage from shoot apex of several adult plants (2 years old) was collected and analyzed. Mucilage from these plants was almost gel like, thus an equal volume of water was needed to extract the soluble fraction for Benedict's test. Slight changes in the color of Benedict's reagent were detected in some samples; however, even in the best case, the A_{700} reading obtained from the mucilage extract translated to about 7 mM of total reducing sugars, which was still much lower than the estimated concentration in the gland tissue of *Gunnera* seedlings (Fig. 2B). These results suggest that *Gunnera* mucilage, especially mucilage from adult plants, may contain reducing sugars but only in limited amounts.

Expression of Genes Encoding Enzymes for the Hydrolysis of Complex Carbohydrates

The mechanism through which simple sugars accumulate in mature glands was investigated by analyzing genes encoding enzymes for starch and Suc hydrolysis identified in a normalized, gland-enriched cDNA library from *G. manicata* (W.-L. Chiu, E.K. Breathwaite, C.R. Secor, and J. Elhai, unpublished data). Sequences of cDNA library (obtained using 2nd-generation DNA sequencing technologies) were screened for genes of interest using the Kyoto Encyclopedia of Genes and Genomes database. The proteins encoded by each cDNA sequence were verified by comparing them to the nonredundant protein database via BLASTX (Altschul et al., 1997). Among cDNA sequences identified (see Table I) were those encoding enzymes that facilitate starch degradation, such as α-amylase (EC 3.2.1.1; an endoamylase that initiates starch degradation), β-amylase (EC 3.2.1.2; an exoamylase that converts amylose to maltose), and a starch phosphorylase (EC 2.4.1.1; an enzyme that catalyzes the reversible transfer of Glc1P to α-1,4-glucan chains). A homolog of STARCH-EXCESS 4 (SEX4), a phosphoglucan phosphatase required for starch degradation (Kötting et al., 2009), was also identified in the gland cDNA library.

Enzymes identified relating to Suc hydrolysis represented in the cDNA library include two invertases (EC 3.2.1.26), a cell wall invertase and a neutral (cytoplasmic) invertase, and a Suc synthase (EC 2.4.1.13; a glycosyltransferase that catalyzes the reversible cleavage of Suc to UDP-Glc and Fru). Also identified in the gland cDNA library was a cDNA encoding a homolog

Table I. *RT-PCR primers for G. manicata cDNA encoding starch/Suc degradation-related proteins*

F, Forward primer; R, reverse primer.

Annotation (GenBank No.)	Predicted Size (Region Covered)[a]	% Positive (Amino Acid)[b]	Primers for RT-PCR (5′ → 3′)
Invertase$_{cell\ wall}$ (HQ009838)	(103–574 amino acids)	79	F:GTCGCTAAGAACCTTGATAGATCAC R:TCTAACCTCTCATAGGTTGGTTCAA
Invertase$_{cytosolic}$ (HQ009839)	571 amino acids	89	F:GCCTGAATACTACGATGGAAAGCTA R:ATCAACAAGTCCAAGACGCAGA
Suc synthase (HQ009840)	811 amino acids	93	F:GGCGAGCTCTATCGTTACATAGC R:CACCTGATCAGGGTGATACGGATC
α-Amylase (HQ009841)	422 amino acids	79	F:GAGTTGTGGCGATTGAAGGATTCG R:GAACAGTGACGGGATTCCAGGAT
β-Amylase (HQ009842)	543 amino acids	76	F:TGTTGGATGATAACGAGGTAGTGG R:GACTCAAGCTACTTGCAAGCTAAC
Starch phosphorylase (HQ009843)	(376–818 amino acids)	80	F:GGCTGCTGCGGATAATGCAT R:TTCTGATGGGACGAGCAAGAG
SEX4 (HQ009844)	381 amino acids	92	F:GCTCCCAACAAAGATGGTCATGTC R:GATGTCCATGCGTTAAGAATACTCTC
Arabinofuranosidase (HQ009845)	690 amino acids	82	F:CACTTCTACAGGATCATCAAAGACTG R:GAAGATATAAGGCTGGAACCTGC
AGP$_{fasciclin}$ (HQ009846)	(24–404 amino acids)	77	F:GCATTCTCCAAAGGCATCGAA R:CTAACGGCTGCGTTCTCGT
AGP$_{classical}$ (HQ009847)	201 amino acids	91	F:GCTCGTACCAATCTCGCC R:ACTTTAGGACCTACATACTGTGGC
TIP41-like protein (HQ009848)	287 amino acids	82	F:ATGGAGAGCCCATTATTCTTCGAG R:CAGACTGATGATGGGAAGCCTC

[a]Coordinates in parentheses refer to those of the protein that best matches the incomplete cDNA sequences from *G. manicata*.   [b]Percentage of positive amino acids compared to the best matched protein obtained through BLASTX.

of Arabidopsis (*Arabidopsis thaliana*) ARAF1, an extracellular α-L-arabinofuronosidase (ARAf; EC 3.2.1.55) capable of catalyzing the hydrolysis of Ara from extracellular polysaccharides such as arabinogalactan-proteins (AGPs) or arabinan-containing pectins (Chávez Montes et al., 2008). The ARAF1 homolog is of interest because it may catalyze the release of Ara from the mucilage.

The expression levels of the above-mentioned genes in the gland and normal stem tissue were analyzed. Total RNA was extracted from stem segments with or without glands, taken from two different batches of *G. manicata* seedlings grown on N-free Murashige and Skoog medium. Reverse transcription (RT)-PCR was performed, using the gene-specific primers listed in Table I. A homolog of type 2A phosphatase activator (TIP41-like protein) was used as an internal control (Expósito-Rodríguez et al., 2008). In general, genes for enzymes involved in the hydrolysis of Suc (Fig. 4, top panel) seemed to be expressed at higher levels than those involved in the breakdown of starch (Fig. 4, middle panel). The high levels of Suc hydrolytic enzymes may explain why N-deprived *Gunnera* plants accumulate mainly Glc and Fru with only a low level of Suc (Fig. 3). Among genes for Suc hydrolytic enzymes, cell wall invertase was expressed at a higher level in stem segments with glands compared to those without. However, both neutral invertase and Suc synthase were expressed at high levels in the stem segments with or without the glands (Fig. 4, top panel).

Among genes encoding enzymes for starch hydrolysis, the expression levels for α-amylase and starch phosphorylase were higher in stem segments with glands compared to those without, whereas β-amylase expression was similar with or without the glands (Fig. 4, middle panel). SEX4, a potential regulator for starch degradation, showed no differences in the expression level regardless of the glands (Fig. 4, bottom panel).

The expression level of ARAf, the extracellular enzyme that may release Ara from AGP or pectin in the mucilage, was slightly higher in the stem segments containing glands than those without (Fig. 4, bottom panel), although the expression level of two genes for AGP core proteins, one classical AGP and one fasciclin-like AGP (Seifert and Roberts, 2007), were the same regardless of the glands (data not shown). However, since the gland sample also contained the neighboring stem tissue, the actual differences in the gene expression levels between stem segments with and without glands are likely underestimated.

Effects of Soluble Sugars on *Nostoc* Differentiation

The initial contact between *Nostoc* and *Gunnera* takes place in the mucilage, which promotes the differentiation of motile hormogonia (Rasmussen et al., 1996). However, once *Nostoc* hormogonia enter *Gunnera* cells, further hormogonia differentiation does not take place. Instead, filaments revert to vegetative cells interspersed
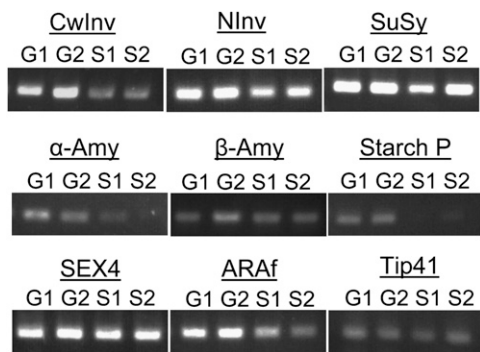
Figure 4. RT-PCR comparison of gene expression in stem segments of N-deprived *G. manicata* seedlings. Expression levels of genes encoding enzymes for the hydrolysis of starch and Suc in stem segments with glands (G1 and G2) and those without glands (S1 and S2) were compared by two-step RT-PCR using total RNA from two different batches of *G. manicata* seedlings grown on N-deprived medium. Abbreviations: CwInv, cell wall invertase; NInv, neutral invertase; SuSy, Suc synthase; α-Amy, α-amylase; β-Amy, β-amylase; Starch P, starch phosphorylase. Tip41 was used as an internal control. Due to the very high expression levels for NInv and SuSy, only 3 μL instead of 5 μL of PCR product for these two genes was loaded on the gel.

with heterocysts. The dramatic differences in the levels of soluble sugars between mucilage and the gland tissue prompted us to test whether soluble sugars can influence *Nostoc* differentiation. In the mucilage, hormogonia differentiation could conceivably be affected by sugars, such as Ara and Gal, released from the hypothetical hydrolysis of AGPs (Rasmussen et al., 1996). As cyanobacteria enter cells of the mature glands, they may encounter Glc, Fru, and Suc. Accordingly, we tested the effect of these soluble sugars on *Nostoc* hormogonia differentiation by streaking a dense culture of *N. punctiforme* onto fresh N-free solid medium supplemented with 30 mM of Ara, Gal, Glc, Fru, Suc, or no sugar.

Upon transfer to fresh N-free media, *Nostoc* filaments differentiated into hormogonia after overnight incubation, regardless of the type of sugar present. They remained as hormogonia for 3 d before reverting back to the N₂-fixing state. After day 3, however, filaments on the Suc plates remained in the vegetative state until day 20, but *Nostoc* on the remaining plates reentered the hormogonia formation cycle by day 7. Subsequently, the extent of hormogonia formation was influenced by the specific sugar in the medium, as indicated by the reduced number of small rod-like hormogonia 3 weeks after the initial hormoglinia induction (Fig. 5, top panels). Arabinose and sugar-free media had a similar effect on hormogonia formation. In both cases, the hormogonia formation cycle continued until the end of the experiment. In a separate batch of experiments, Fru was found to inhibit secondary hormogonia formation to the same extent as Glc (data not shown). Overall, the inhibitory effect of sugars on hormogonia differentiation was in the order of Suc > Glc/Fru > Gal > Ara. The measurements of

hormogonia frequency were corroborated by observations of the density of growth on the medium 5 weeks later, which was affected by the presence of sugar in the same way as hormogonia differentiation (Fig. 5, bottom panels). *Nostoc* on medium with Ara or without sugar did not grow as well, presumably because frequent hormogonia formation prevented heterocyst differentiation required for growth on N-free medium.

DISCUSSION

To establish productive associations with N₂-fixing cyabanocteria, plant hosts must solve the problems of attracting and internalizing cyanobacteria and regulating cyanobacterial growth and differentiation once the cyanobionts are inside the plant. This study took advantage of the N-deprivation-induced gland development in *G. manicata* (Chiu et al., 2005) to identify factors that may enable *Gunnera* plants to establish endosymbiosis with *Nostoc*.

Similar to other plants under chronic N stress (Hermans et al., 2006; Bi et al., 2007), *G. manicata* seedlings grown on N-deprived medium accumulated a large amount of starch in the stem (Fig. 1). In contrast, cells in the mature glands were nearly starch free (Fig. 1, D and F). Instead, mature glands accumulated high levels of soluble sugars, especially Glc and Fru (Fig. 3A), compared to normal stem tissue (Fig. 2B). Glc and Fru have been shown to support nitrogenase activities in *Nostoc* strains associated with *Anthoceros* (Steinberg and Meeks, 1991) and *Gunnera* (Man and Silvester, 1994; Wouters et al., 2000). In stem segments with mature glands, the total concentration of Glc and Fru was around 7 mM (Fig. 3A). As the plants grew older, the level of these sugars in uncolonized stems continued to increase (Fig. 3B). However, the actual levels of soluble sugars may be even higher since the estimated concentration of total reducing sugars in stems with mature glands, using Benedict's test (Fig. 2B), was between 20 to 25 mM. Hence, the level of metabolizable sugars in *Gunnera* stems prior to *Nostoc* colonization is likely higher than the 10 mM Fru used to sustain long-term nitrogenase activity in darkness for *Nostoc* PCC 9229, a strain originally isolated from *Gunnera monoica* (Wouters et al., 2000). Successful *Nostoc* colonization drastically reduced Glc and Fru accumulation in the colonized portion of the stem (Fig. 3B), presumably because most sugars were consumed to support N₂ fixation and amino acid synthesis. This is consistent with the observation that *Gunnera* plants grown on N-replete medium did not accumulate reducing sugars (Fig. 2A).

As one would expect from the high Glc and Fru levels in N-stressed *G. manicata* plants (Fig. 3), several genes encoding Suc-cleaving enzymes, including cell wall invertase, neutral (cytoplasmic) invertase, and Suc synthase, were expressed at very high levels in the stem, especially the latter two (Fig. 4, top panel). Expression of genes encoding enzymes for starch
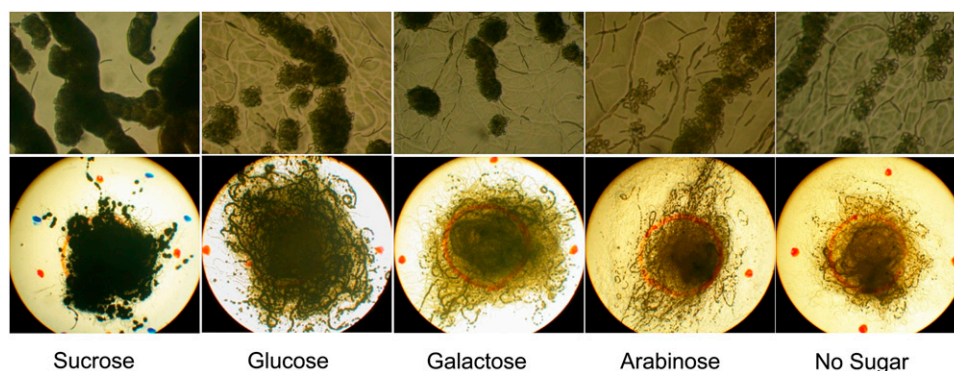
Sucrose   Glucose   Galactose   Arabinose   No Sugar

Figure 5. The effects of soluble sugars on *N. punctiforme* hormogonia differentiation. *N. punctiforme* was induced to form hormogonia (i.e. the rod-shaped filaments in the top panels) by streaking cells from a dense culture onto fresh N-free solid medium supplemented with 30 mM of the indicated sugar or with no sugar. Streaking was confined to the center of the plate, marked by a red circle (1 cm in diameter) visible in some bottom panels. *Nostoc* filaments differentiated into hormogonia after overnight incubation on fresh medium; however, the frequencies of subsequent hormogonia formation varied, depending on the specific sugar present in the media. Pictures of the culture were taken under a stereo microscope 3 weeks (top panels) or 5 weeks (bottom panels) after initial hormogonia induction. The magnifications of the top and bottom panels were approximately 64× and 7.5×, respectively. The red and blue dots on the bottom panels were used to mark the extent of hormogonia movement at the end of the first week.

degradation could also be detected, but their RNA was not as abundant compared to those for Suc hydrolysis (compare Fig. 4 top and middle panels). Starch phosphorylase and α-amylase were expressed at higher levels in the stem segments with glands (Fig. 4, middle panel), which may be partially responsible for the lack of starch accumulation in the glands. Increased starch and Suc breakdown through higher expression of starch and sugar degradation enzymes were also observed in rhizobium-colonized nodules (Colebatch et al., 2004; Flemetakis et al., 2006; Tesfaye et al., 2006). Identifying the mechanisms through which plants regulate expression of genes for starch and Suc hydrolysis enzymes in the symbiotic tissue is crucial to understand the ability of the plant tissue to host N_2-fixing microbes.

Despite high levels of soluble sugars in gland tissue, sugar levels in the mucilage collected on the gland surface were barely detectable. Earlier analysis of mucilage from *Gunnera chilensis* indicated that carbohydrates in the mucilage are mainly polymers of Ara, Gal, and GlcUA in the ratio of 1.00:0.25:0.13 (Rasmussen et al., 1996). The same study suggested that polysaccharides in the *Gunnera* mucilage are mainly arabinogalactans associated with AGPs (Rasmussen et al., 1996). Through analysis of glycosidic linkage composition, AGPs were also implicated as the primary component of mucilage secreted by pea (*Pisum sativum*) roots (Knee et al., 2001). However, the ratio of Ara to Gal in legume root mucilage is close to 1:1 (Knee et al., 2001), quite different from the 4:1 ratio found in the *Gunnera* mucilage (Rasmussen et al., 1996). The higher Ara to Gal ratio may be due to arabinan-containing pectins in the *Gunnera* mucilage in addition to AGPs. Since breakdown of the middle lamella takes place in the mature glands (Towata, 1985), it is rea-

sonable to have some remnant of pectin from the middle lamella appear in the mucilage. The Ara in the mucilage may be released through an ARAf encoded by a homolog of Arabidopsis gene ARAF1, which can use pectin as substrate (Chávez Montes et al., 2008). This ARAf is expressed at a higher level in stem segments containing glands (Fig. 4, bottom panel). The presence of Ara in the mucilage may be of functional significance because Ara was found to be more effective than Glc or Gal in attracting *Nostoc* hormogonia, although none of these simple sugars was as effective as root extract from white clover (*Trifolium repens*) or mucilage from *G. manicata* (Nilsson et al., 2006).

If free Ara and Gal in the mucilage is sufficient to attract *Nostoc* (Nilsson et al., 2006), they should not interfere with the *Nostoc* hormogonia formation cycles (Fig. 5). This is important because the ability to form hormogonia is required before *Nostoc* filaments enter plant cells. Once inside the *Gunnera* cells, however, hormogonia formation must be prevented for N_2 fixation to take place. The abundance of Glc and Fru in tissue associated with mature glands may serve to inhibit hormogonia formation and promote *Nostoc* heterocyst differentiation and N_2 fixation.

The inhibition of hormogonia formation by Glc and Fru may be mediated by the action of a water-soluble hormogonia repression factor similar to that produced by the hornwort *Anthoceros punctatus* (Meeks and Elhai, 2002). Hormogonia repression factor induces genes that repress hormogonia formation through inactivation of a transcriptional repressor HrmR (Campbell et al., 2003). HrmR belongs to the LacI/GalR family of sugar-binding repressors and binds galacturonate in vitro (Campbell et al., 2003). According to the model proposed by Campbell et al. (2003), HrmR would be sugar bound and inactive in the high sugar

concentrations found inside of *Gunnera* cells, thus allowing genes that negatively regulate hormogonia formation to be expressed.

Taken together, results from this study suggest that, in an N-limiting environment, *Gunnera* glands secrete mucilage with very low levels of soluble sugars, which help to attract *Nostoc* to the surface of the gland but does not interfere with the hormogonia formation cycle. From there, hormogonia travel into the gland and enter cells at the base of the gland most likely because they are attracted by the high levels of soluble sugars inside these cells. Once inside plant cells, further hormogonia formation is prevented by the high levels of sugars in these cells. Understanding how the expression of genes encoding starch/Suc degradation enzymes is up-regulated in the tissue associated with mature glands will be crucial to understanding the ability of *Gunnera* tissue to provide an environment for the establishment of endosymbiosis with cyanobacteria.

MATERIALS AND METHODS

Cultivation of *Nostoc* Punctiforme

Nostoc punctiforme ATCC 29133 was obtained from Jack Meeks (University of California at Davis) and cultivated on N-free medium according to Chiu et al. (2005). To test the effect of various sugars on hormogonia formation, a small amount of *N. punctiforme* from a 3- to 4-week-old N-free plate-grown culture was streaked out onto the center (about 5 mm in diameter) of a 60×10 mm petri plate containing fresh N-free solid medium supplemented with 30 mM of one of the following sugars: Suc, Glc, Fru, Gal, or Ara. Prior to autoclaving, each medium was buffered with 0.5 mM MES (pH 5.8). All media were solidified with 0.6% (w/v) Gellan Gum (Caisson Laboratories). Cultures were maintained at 25°C under a 16-h/8-h photoperiod at 50 μmol m^{-2} s^{-1}. The cultures were observed for 5 weeks with data recorded every other day. Pictures of the cultures were taken using an Olympus SZH stereo microscope.

Cultivation and Manipulation of *Gunnera manicata* Seedlings

Germination of *Gunnera manicata* seeds was as described by Chiu et al. (2005). Seedlings were maintained in 0.5× Murashige and Skoog medium supplemented with 0.5% (w/v) Suc. To induce gland development, seedlings were transferred to N-free Murashige and Skoog medium (PhytoTechnology Laboratories) without sugar and grown at 25°C under constant light at 30 μmol m^{-2} s^{-1}. To induce gland development in soil-grown plants, plate-grown seedlings were transferred to 42 mm Jiffy-7 peat pellets (Jiffy Products), irrigated with tap water, and maintained in a growth chamber at 25°C under a 16-h/8-h photoperiod at 40 μmol m^{-2} s^{-1}. Under this growth condition, glands begin producing mucilage after about 1 month.

Detection of Starch and Reducing Sugars in *Gunnera* Tissue

Starch was detected by adding a drop of a solution containing 1% w/v each of I$_2$ and KI onto fresh sections of *Gunnera* seedling stems. For reducing sugar assays, approximately 30 mg of *Gunnera* tissue was weighed and frozen in liquid N. The samples were then ground up in 60 μL of Benedict's reagent in a microfuge tube and incubated at 95°C for 5 min. The samples were cleared by centrifugation, and the remaining blue color of Cu^{2+} in Benedict's reagent was quantified by measuring the absorption at 700 nm (A_{700}; Cochran et al., 2008) using a NanoDrop spectrophotometer (Thermo Scientific). A_{700} reading for each sample was normalized against the sample weight. Since the disappearance of blue color depends on reducing sugars in the homogenate, the amount of reducing sugars in each sample is negatively correlated to A_{700}.

The concentration of total reducing sugars in each sample was estimated according to a standard curve constructed using known concentrations of Glc solutions.

Quantitative Analysis of Soluble Sugars in *Gunnera* Tissue

Stem segments of *G. manicata* were weighed, frozen in liquid N, and stored in liquid N until use. To extract soluble sugars, stem segments weighing between 100 and 200 mg were ground to a fine powder in liquid N using a mortar and pestle. The powder was suspended in 2.5 mL of 80% ethanol, and the mixture was heated to 80°C for 1 h with occasional vortexing. After a brief centrifugation, the supernatant was collected. The ethanol extraction procedure was repeated once. The supernatant was then dried in 100 μL aliquots using a SpeedVac concentrator (Thermo Scientific) and reconstituted in water. The concentrations of Glc, Fru, and Suc were measured using an enzymatic NADP-reducing method (UV method) kit from Roche based on Bergmeyer and Bernt (1974). In brief, Glc and Fru in the sample were first phosphorylated by hexokinase to yield Glc-6-P (G-6-P) and Fru-6-P. Subsequently, in the presence of G6P dehydrogenase, G-6-P was detected by its ability to reduce NADP$^+$ to NADPH. Fru-6-P was converted to G-6-P by phosphoglucose isomerase and Suc was hydrolyzed to Fru and Glc by invertase before they were measured. The amount of NADPH produced was measured by reading the A_{340} using a NanoDrop spectrophotometer (Thermo Scientific).

RNA Isolation and RT-PCR Analysis of Gene Expression

Stems of *G. manicata* seedlings grown on N-free Murashige and Skoog medium with mature glands were divided into two segments, one with glands (the upper half) and one without glands (the lower half) and immediately frozen in liquid N. Total RNA was isolated from these samples using TriZol RNA reagent (Molecular Research Center). First-strand cDNA synthesis was carried out using SuperScript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. First-strand cDNA was diluted 5-fold and used as a template for PCR. PCR was carried out using GoTaq Green Master Mix (Promega) and gene-specific primers listed in Table I. The PCR conditions were 30 s at 94°C, 30 s at 55°C, 30 s at 72°C, and 5 min at 72°C for 35 cycles with 3 min at 94°C to start up.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers listed in Table I.

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