Nitrogen Deprivation Stimulates Symbiotic Gland Development in *Gunnera manicata*[1]

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Gunnera is the only genus of angiosperms known to host cyanobacteria and the only group of land plants that hosts cyanobacteria intracellularly. Motile filaments of cyanobacteria, known as hormogonia, colonize Gunnera plants through cells in the plant's specialized stem glands. It is commonly held that Gunnera plants always possess functional glands for symbiosis. We found, however, that stem gland development did not occur when *Gunnera manicata* plants were grown on nitrogen (N)-replete medium but, rather, was initiated at predetermined positions when plants were deprived of combined N. While N status was the main determinant for gland development, an exogenous carbon source (sucrose) accelerated the process. Furthermore, a high level of sucrose stimulated the formation of callus-like tissue in place of the gland under N-replete conditions. Treatment of plants with the auxin transport inhibitor 1-naphthylphthalamic acid prevented gland development on N-limited medium, most likely by preventing resource reallocation from leaves to the stem. Optimized conditions were found for in vitro establishment of the Nostoc-Gunnera symbiosis by inoculating mature glands with hormogonia from *Nostoc punctiforme*, a cyanobacterium strain for which the full genome sequence is available. In contrast to uninoculated plants, *G. manicata* plants colonized by *N. punctiforme* were able to continue their growth on N-limited medium. Understanding the nature of the Gunnera plant's unusual adaptation to an N-limited environment may shed light on the evolution of plant-cyanobacterium symbioses and may suggest a route to establish productive associations between N-fixing cyanobacteria and crop plants.

Nitrogen (N)-fixing cyanobacteria enter into symbiotic associations with a phylogenetically diverse group of eukaryotes ranging from fungi to vascular plants (Rai et al., 2000). Gunnera is the only flowering plant genus known to host cyanobacteria (Bonnett, 1990; Bergman et al., 1992; Bergman and Osborne, 2002). Plants in the genus Gunnera are mainly distributed in the southern hemisphere with about 40 species, which includes small, stoloniferous species (e.g. *Gunnera magellanica*) and plants 6 to 8 ft in height (e.g. *Gunnera manicata*). Even in the latter case, N_2 fixed by the symbiotic cyanobacteria, most likely in the form of NH_3 (Silvester et al., 1996), is able to fulfill the N needs of the plant (Osborne et al., 1991, 1992).

In nature, Gunnera plants form symbioses with cyanobacteria belonging to the genus Nostoc (Bergman et al., 1992). Unlike other multicellular plant hosts, Gunnera harbors Nostoc intracellularly, surrounding the Nostoc filaments with a membrane most likely of plasma membrane origin (Silvester and McNamara, 1976; Johansson and Bergman, 1992). The specialized glands on the Gunnera stem serve as entry points for Nostoc filaments. Gunnera glands are conspicuously red due to the accumulation of anthocyanins such as pelargonidin and cyanidin (G. Lanigan, K.G. Black, and B. Osborne, unpublished data). Shortly after germination, the origins of the glands may be noticeable on some seedlings as organized red spots on the surface of the hypocotyl just below the point of cotyledon attachment (Bergman and Osborne, 2002). Subsequently, one new gland typically appears on the stem at the base of each new leaf. Under natural conditions, all of the glands are colonized by cyanobacteria (Bonnett, 1990; Osborne et al., 1991).

Anatomical studies of several different Gunnera species have determined that a gland originates from a group of cells within the stem that resume mitotic activities after seedling establishment (Bonnett, 1990; Johansson and Bergman, 1992). Each progenitor cell produces an independent multicellular papilla that extends externally, eventually rupturing the epidermal layer. These papillae are organized in a circular pattern with a central papilla surrounded by five to eight others. This organization creates multiple channels around the papillae that reach into the stem without directly exposing cells to the outside environment. Nostoc filaments, in the form of motile hormogonia, enter Gunnera plants through cells lining these channels (Johansson and Bergman, 1992; Uheda and Silvester, 2001). Cells on the surface of the channels are cytoplasm rich and may secrete carbohydrate-rich mucilage, which can support the growth of cyanobacteria

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as well as other microbes on the gland (Bonnett, 1990; Bergman et al., 1992; Johansson and Bergman, 1992; Rasmussen et al., 1994). Furthermore, these cells can continue to divide after infection to form regions of symbiotic tissue in the stem. Investigation of the genesis and physiology of the stem glands may provide insight into molecular characteristics that allow plant cells to host cyanobacteria.

The ability to form hormogonia is a prerequisite for a cyanobacterial strain to colonize Gunnera stem glands (Bonnett and Silvester, 1981; Johansson and Bergman, 1992, 1994). Hormogonia can be induced by environmental- as well as plant-derived factors (Campbell and Meeks, 1989; Rasmussen et al., 1994; Meeks and Elhai, 2002). Since hormogonia are incapable of vegetative growth, they usually revert back to the vegetative state after 48 to 72 h. Previous studies have demonstrated that it is not necessary to use a culture of cyanobacteria with pre-existing hormogonia to successfully colonize Gunnera (Silvester and McNamara, 1976; Johansson and Bergman, 1992, 1994), but a culture rich in hormogonia can greatly increase the efficiency of infection (Uheda and Silvester, 2001).

Virtually all molecular research to date on plant-cyanobacterial associations has focused on the cyanobacterial partner. A major reason for this bias is that symbiotic cyanobacteria (mainly Nostoc species) have proven easy to grow and manipulate genetically in the laboratory (Meeks and Elhai, 2002). Recently, the genome of a symbiotic strain of Nostoc, *Nostoc punctiforme*, has been completely sequenced (Meeks et al., 2001; J.C. Meeks, unpublished data). In order to facilitate the molecular characterization of cellular interactions between *N. punctiforme* and Gunnera plants, we have developed a simple protocol for routine establishment of the symbiosis between Nostoc and *G. manicata* in aseptic culture. In the process, we discovered that Gunnera stem glands could not develop unless the plant was deprived of exogenous combined N.

RESULTS

Growth and Vegetative Propagation of *G. manicata* in Culture

In order to study the Nostoc-Gunnera symbiosis under defined conditions, it is necessary to use plants maintained in aseptic culture. Plant culture media lacking combined N have been used for the culture of *G. manicata* seedlings up to the cotyledon stage immediately prior to inoculation with cyanobacteria (Johansson and Bergman, 1994; Uheda and Silvester, 2001). However, there are drawbacks when experiments are restricted to the use of seed-derived material. First, *G. manicata* seeds do not germinate well after extended storage. We saw a more than 70% reduction in the germination rate of seeds from the same source within one year. Second, the glands on *G. manicata* seedlings were only transiently receptive to cyanobacteria infection, and the first pair of glands on seedlings more than 2 months old could not be infected, in agreement with previous results (Johansson and Bergman, 1992; Uheda and Silvester, 2001). For these reasons, it was desirable to establish a procedure to maintain plant material through vegetative propagation in the absence of cyanobacteria. This required a medium containing combined N.

We found *G. manicata* seeds germinated well and seedlings developed healthy leaves on medium containing half-strength Murashige and Skoog salts (including ammonium nitrate; Murashige and Skoog, 1962), supplemented with 1% Suc. However, primary root growth on this medium was very poor, seldom exceeding 1 cm in length. Since half-strength Murashige and Skoog-salts medium contains a very high concentration of combined N (almost 30 mM), we tested other media containing lower salt concentrations. A plant maintenance medium (PMM) modified from one commonly used to grow Arabidopsis (*Arabidopsis thaliana*) seedlings (Nutrient Solution; Haughn and Somerville, 1986) containing 10 mM combined N permitted seed germination and the development of seedlings with healthy shoots and roots. Except for the inclusion of 10 mM KNO_3, this medium has a salt concentration similar to the N-free half-strength Hoagland solution employed by Uheda and Silvester (2001).

Outgrowth of axillary buds on *G. manicata* can be initiated by removing the apices from 1- to 2-month-old plants to release apical dominance. Shoots derived from axillary buds were removed and transferred to fresh PMM medium. Roots usually developed on these plants within 2 weeks after the transfer. As illustrated in Figure 1A, vegetative propagation can yield healthy plants in just a few weeks.

Nitrogen Regulation of Symbiotic Gland Development

Due to the accumulation of anthocyanins, the location of the glands is frequently visible on Gunnera seedlings upon germination (Bergman and Osborne, 2002). To our surprise, there was no trace of the gland on more than a hundred healthy plants, each derived from the axillary bud of an individual seedling, maintained on solidified medium containing 10 mM nitrate for up to 3 months. On the other hand, the anthocyanin-marked outline of the gland started to appear on the stem at the base of each leaf petiole within a few weeks after these plants were transferred from N-replete medium to N-free medium. Gland development on these plants appeared to be synchronized. Most of the glands on the same plant reached maturity in about 2 months, when they appeared bright red (Fig. 1B) and covered by a layer of mucus (Fig. 1B, insert). The glands developed in culture had the typical organization of a Gunnera gland: a central papilla surrounded by eight others (Fig. 1B, insert).

To investigate the relationship between the onset of gland development and nutrient status, we tested the effects of exogenous N and carbon (C) on seedling

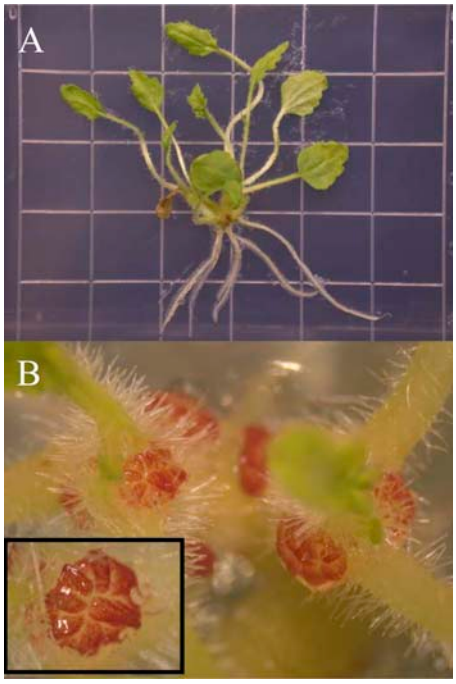

Figure 1. *G. manicata* plants derived from in vitro culture of axillary buds. A, Five weeks after detached buds were transferred to fresh medium containing 10 mM nitrate. B, Bright red glands that developed on the stem at the base of each leaf petiole about 1 month after vegetatively propagated plants were transferred to N-free medium. Insert shows a closer view of a mature gland covered with mucus.

gland development. Young seedlings were used for these experiments instead of vegetatively propagated plants because it is easier to obtain a large number of seedlings that are at the same developmental stage. Newly germinated *G. manicata* seedlings were transferred to medium containing all combinations of 0, 0.1, and 10 mM nitrate and 0, 0.5%, and 2.5% Suc. On average, 15 seedlings were used for each Suc/nitrate combination. The presence or absence of glands and the number of live plants on each medium after 2 months are listed in Table I. Consistent with previous results, of the seedlings on these nine different media, only those on media containing no nitrate ($n = 36$) or 0.1 mM nitrate ($n = 35$) developed typical glands on the hypocotyls. Although on some seedlings the origins of the hypocotyl glands were visible upon germination as organized spots of anthocyanin accumulation (e.g. Fig. 2A), gland development per se did not proceed when these seedlings were maintained on media containing 10 mM nitrate ($n = 41$; Table I). However, when recently germinated seedlings were transferred to N-free or N-limited (0.1 mM nitrate) medium, a typical gland replete with anthocyanins formed within a few weeks (Fig. 2B).

G. manicata seedlings maintained on N-replete medium until two to three true leaves had formed and then transferred to 0.1 mM nitrate or N-free nutrient agar formed more pronounced glands on the hypocotyls (Fig. 2C) than those formed on younger seedlings. Evidently, given an adequate endogenous nutrient supply, gland cells do in fact have the potential to divide and form the symbiotic structure independent of the presence of cyanobacteria, as was previously suggested (Bonnett, 1990; Johansson and Bergman, 1992).

Effects of Suc on Gland Development

Although the initiation of gland development was triggered mainly by N deprivation, the presence of moderate concentrations of Suc (0.5%–1%) on N-limited media accelerated gland development both in seedlings and in vegetatively propagated *G. manicata* plants. However, seedlings on N-free or N-limited medium supplemented with a high concentration of Suc (2.5%) accumulated a copious amount of anthocyanins throughout the hypocotyls (e.g. Fig. 2D), and their growth was inhibited ($n = 31$).

The negative effect of high Suc on seedling growth was attenuated when the concentration of nitrate in the medium was increased to 10 mM ($n = 12$). No typical gland development occurred on this nutrient-rich medium (10 mM nitrate and 2.5% Suc). Surprisingly, after growing on this high Suc medium for more than 2 months, callus-like tissue started to appear on the hypocotyl in place of the glands (Fig. 2, E and F). Besides lacking an organized gland structure, the callus-like growth also lacked the bright red color of a typical gland, appearing brownish instead.

Inhibition of Gland Development by an Auxin Transport Inhibitor

Gunnera stem gland development is accompanied by the initiation of cell division, a process in plants that usually requires auxin. Also, adventitious root formation, an auxin-dependent process, tends to occur immediately below the gland (data not shown). These observations suggest that the site of gland development may accumulate elevated levels of auxin under N-limited conditions.

In order to test whether auxin plays a role in gland development induced by N deprivation, *G. manicata*

Table I. *Gland development in seedlings grown on different levels of Suc and nitrate*

The symbols + and − indicate the presence and absence of gland, respectively, at the end of 2 months. The asterisk (*) indicates that callus-like tissue formed where glands would have been expected to appear. The number of plants observed in each condition is shown in parentheses.

Nitrate	Sucrose		
	0	0.5	2.5
mM		%	
0	+ (10)	+ (14)	+ (12)
0.1	+ (7)	+ (9)	+ (19)
10.0	− (14)	− (14)	* (12)

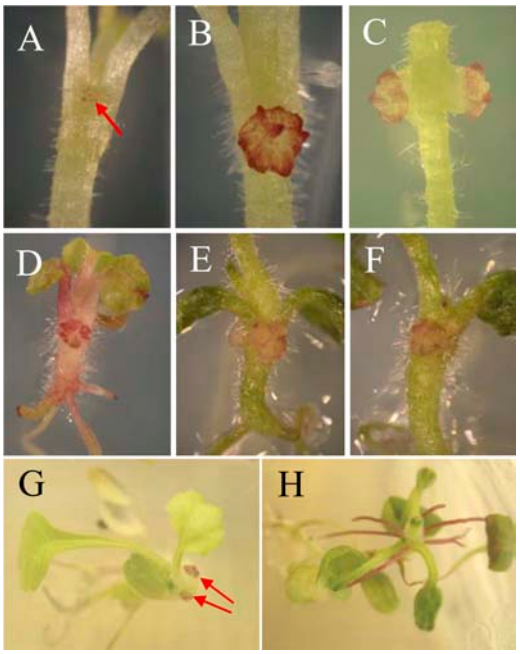

Figure 2. Gland development on *G. manicata* seedlings. Glands of seedlings germinated on N-replete medium and then grown for 6 weeks on the medium containing 10 mM (A) or 0.1 mM (B) nitrate. C, Hypocotyl glands on seedlings initiated on medium containing nitrate for 1 month and then transferred to N-free medium for an additional month. D, A seedling that accumulated high levels of anthocyanins after growing on N-free medium supplemented with 2.5% Suc for 2 months. E and F, Callus-like tissue which formed on the hypocotyl of a 2-month-old seedling growing on medium containing 10 mM nitrate and 2.5% Suc. G and H, Healthy *G. manicata* seedlings with four true leaves after transfer to N-free medium (G) or N-free medium plus 50 μM auxin-transport inhibitor NPA (H) for 2 months. Note that NPA-treated seedlings formed adventitious roots instead of glands and that their leaves remained green on N-free medium. Arrows indicate the positions of glands (G) or where a gland would be expected (A).

seedlings grown on N-replete medium until four true leaves had formed were transferred to N-free medium containing 0, 5, or 50 μM of the auxin efflux inhibitor 1-naphthylphthalamic acid (NPA). Plants on N-free medium without NPA turned pale green and initiated gland development within several weeks, as expected (Fig. 2G). However, leaves of plants on 5 or 50 μM NPA stayed dark green for 3 months on N-free medium. Eventually, traces of red pigments at the sites for the glands were visible on the upper portion of the plants grown on 5 μM NPA ($n = 17$), but they were absent from plants grown on 50 μM NPA ($n = 17$). Instead, numerous red-colored adventitious roots developed on the stem of plants treated with 50 μM NPA (Fig. 2H).

Establishment of Symbiosis in Vitro

In order to test the function of Gunnera glands developed in culture, efforts were made to establish symbiosis using these glands and the cyanobacterium *Nostoc punctiforme*. Rapid hormogonia formation was found to be induced by diluting a sample of *N. punctiforme* taken from a well established culture on an N-free PMM plate and replating it on fresh N-free PMM (see "Materials and Methods"). Under these conditions, essentially all Nostoc vegetative filaments (Fig. 3A) differentiated into hormogonia during an overnight incubation (Fig. 3B). Diluting and plating of a culture onto fresh medium is known to induce hormogonia formation in other cyanobacterial strains (Hernández-Muniz and Stevens, 1987; Herdman and Rippka, 1988).

Newly formed hormogonia of *N. punctiforme* were very active and able to move around on the plate leaving visible tracks on the surface of the solid medium (Fig. 3B). These tracks most likely represent the deposit of polysaccharide slime secreted by the Nostoc cells, which may facilitate the movement of hormogonia (Hoiczyk and Baumeister, 1998; Hoiczyk, 2000). As

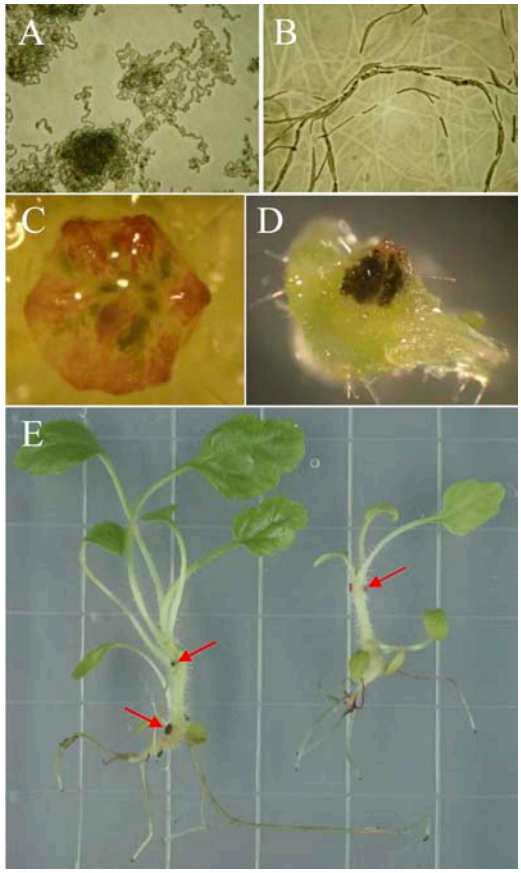

Figure 3. Establishment of Gunnera-Nostoc symbiosis in vitro. A, Vegetative filaments of *N. punctiforme* growing on solid N-free plant maintenance medium. B, Hormogonia of *N. punctiforme* that formed overnight after diluting vegetative filaments on solid medium from a culture shown in A. Note tracks formed by moving hormogonia. C, Growth of *N. punctiforme* on the surface of a mature gland 3 d after inoculating the gland with hormogonia. D, Cross section of the stem of a colonized *G. manicata* plant that revealed Nostoc colonies (dark color) beneath a stem gland. E, Growth of *G. manicata* seedlings on N-limited medium with (left) or without (right) symbiotic *N. punctiforme* for 2 months. Arrows show the positions of glands.

shown in Figure 3B, it is very common to see several hormogonium filaments lining up on the same track either as single filaments or in bundles of filaments. For this reason, streams of hormogonia on solid medium, viewed macroscopically, often give the appearance of very long filaments.

The occurrence of dark green Nostoc patches on the surface of susceptible glands (Fig. 3C) 3 d after the inoculation with hormogonia indicates that at least some hormogonia were able to revert to vegetative growth soon after they were placed on the glands. Two months later, the beneficial effects of Nostoc colonization on the host plants were readily apparent. While the uninoculated seedling appeared pale green and hardly changed in size on N-limited medium (0.1 mM NO_3), *G. manicata* seedlings colonized by *N. punctiforme* showed healthy growth on the same medium (Fig. 3E), even though the volume of the colonized tissues was very small compared to that of the rest of the plants.

The dark green area of Nostoc colonization directly behind the gland is apparent in a cross section of a stem (Fig. 3D). Nostoc-colonized cells could be seen from near the surface of the gland all the way across the cortex to the edge of the stele. Nostoc filaments from this colonized gland were recovered simply by cutting up the dark green tissue and placing it on an N-free PMM plate. Despite the fact that vegetative growth is limited and there is no hormogonia formation when Nostoc filaments are in the symbiotic environment (Meeks and Elhai, 2002), Nostoc filaments recovered from the *G. manicata* tissue were able to resume growth and spontaneously differentiate into hormogonia on the N-free plate soon after they were released from the host cells.

DISCUSSION

Gunnera plants enter into productive intracellular symbioses with a variety of Nostoc species (Johansson and Bergman, 1994) including *N. punctiforme*, which also can form an extracellular symbiosis with the hornwart *Anthoceros punctatus* (Enderlin and Meeks, 1983). The strain was originally isolated from an extracellular symbiosis with a third plant, the cycad *Macrozamia* sp. (Rippka et al., 1979). Plant-cyanobacterial symbioses are generally less specific with respect to the Nostoc partner (Meeks and Elhai, 2002), contrasting with the stringent specificity required for most plant-rhizobial symbioses (Riely et al., 2004). The promiscuity of symbiotic Nostoc, among other reasons, has led to the postulate that plant hosts, not cyanobacteria, bear the primary responsibility for determining whether a symbiosis can take place (Meeks, 1998; Meeks and Elhai, 2002).

Plant-cyanobacterial symbioses also differ from plant-rhizobial symbioses in that the colonized structure in the former develops independently of cyanobacterial infection (Bonnett, 1990; Johansson and Bergman, 1992). Since stem glands are always found on Gunnera plants growing in their natural habitat (Silvester and McNamara, 1976; Bergman and Osborne, 2002), it has been presumed that glands form constitutively and gland development is part of the normal growth for Gunnera plants (Bonnett, 1990; Rai et al., 2000; Bergman and Osborne, 2002; Meeks and Elhai, 2002). In the case of *G. manicata*, we have shown that this is not true. Indeed, the position of the glands is predetermined; when present, they always occur on each side of the hypocotyl and on the stem right below the base of the leaf petiole. However, gland development is specifically induced in response to N deprivation (Figs. 1 and 2). Previous works may have overlooked this requirement because of the frequent appearances of red-marked hypocotyl gland origins independent of the N status. In this regard, Gunnera-Nostoc symbiosis parallels the situation in legume-rhizobia symbiosis since exogenous combined N inhibits early interactions between legume and rhizobia and prevents symbiotic nodule formation (Limpens and Bisseling, 2003). N-dependent phenomena have also been demonstrated in the symbiosis between Nostoc and the hornwort *A. punctatus*. Only when Anthoceros is deprived of N does it produce a compound that induces hormogonia formation in Nostoc (Campbell and Meeks, 1989). N deprivation is also required for Anthoceros to produce signals for maintaining high N_2-fixing ability in the associated Nostoc (Campbell and Meeks, 1992).

While N deprivation is the major factor regulating stem gland development, the availability of sugar can also affect gland development. In general, gland development was accelerated on N-limited medium supplemented with a modest level of Suc (0.5% to 1%). Furthermore, a high level of exogenous Suc (2.5%) could partially overcome the inhibitory effect of combined N (10 mM nitrate) on gland development but resulted in callus-like tissue instead of a well organized gland (Fig. 2, E and F). High levels of Suc are known to promote plant cell proliferation through activation of cyclin D genes (Riou-Khamlichi et al., 2000). One of these cyclin genes, *cycD3*, is normally expressed in proliferating tissues, and constitutive expression of this gene caused hyperproliferation of leaf cells and interfered with their differentiation (Dewitte et al., 2003). The callus-like tissue replacing the glands on plants grown under nitrate-replete conditions is most likely the result of hyperproliferation of gland initial cells triggered by the high level of Suc.

Judging from the dramatic differences of *G. manicata* seedlings grown on media containing various combinations of nitrate and Suc (Fig. 2, A–F), *G. manicata* is extremely sensitive to changes in the C:N ratio. It is known from studies in Arabidopsis that the C/N-sensing ability of plants can activate genes involved in N assimilation when the supply of C skeletons is abundant (Coruzzi and Zhou, 2001). In Gunnera, the C/N-sensing mechanism may play an important role in regulating the initiation of gland development in order to establish an effective N_2-fixing symbiosis.

How are changes in N status linked to the regulation of gland development? Judging from the fact that the auxin transport inhibitor NPA prevented both the degradation of chlorophyll in leaves and the development of stem glands in N-deprived *G. manicata* plants (Fig. 2H), normal auxin movement from shoot to root appears to be required for reallocation of resources from leaves to the stem to trigger gland development. Auxin is synthesized in shoots and transported to roots. For this and other reasons, auxin was put forth as a good candidate to communicate the N status of the shoot to roots (Forde, 2002). The observations in *G. manicata* lend support to this hypothesis.

Gunnera is peculiar in that it is the only genus of angiosperms known to establish N_2-fixing symbioses with cyanobacteria. The unique structure and properties of the stem glands are crucial for the successful association between Gunnera and cyanobacteria. Although the mucilage secreted by the gland can attract and support the growth of Nostoc as well as other bacteria on the surface of the gland (Johansson and Bergman, 1992), only Nostoc is able to enter Gunnera cells. We observed the establishment of Nostoc on the mucilage-covered surface of mature glands 3 d after inoculating the gland with hormogonia (Fig. 3C). Nostoc filaments were concentrated mainly in the valleys of the gland, which coincide with the opening of the channels that lead to internal portions of the gland. Hence, hormogonia formed from these vegetative filaments are well positioned to invade the cells lining the surface of the channels. Since hormogonia have a tendency to follow preexisting tracks on solid surface (Fig. 3B), the attraction of one hormogonium filament into the channels may lead to efficient recruitment of many others.

It has been suggested that coevolution between Gunnera and cyanobacteria led to the formation of the stem glands (Chapman and Margulis, 1998). Understanding the cellular and molecular basis of symbiotic gland development and the mechanisms underlying the ability of Gunnera cells to maintain and exploit N_2-fixing cyanobacteria may shed some light on the evolutionary path leading to this unique intracellular symbiosis and may provide us with knowledge necessary for the establishment of novel symbioses between cyanobacteria and crop plants.

MATERIALS AND METHODS

Cultivation of *Gunnera manicata*

Seeds of *Gunnera manicata* were obtained from a commercial nursery (Hortus Botanicus) and stored at room temperature for use within 12 months. Prior to germination, seeds were rinsed with 70% ethanol and then soaked for 20 min in 3% sodium hypochlorite. After several rinses with sterile deionized water, seeds were kept in water in a sterile beaker covered with foil and placed on an orbital shaker (120 rpm). The seeds were incubated until their outer coats fell off, a process that took a week to 10 d. The sterilization step with sodium hypochlorite and rinsing was repeated two to three more times during the incubation.

Sterilized seeds were placed on PMM modified from Haughn and Somerville (1986) and containing the following: 5 mM KNO_3; 2.5 mM KH_2PO_4; 2 mM $MgSO_4$; 2.5 mM $Ca(NO_3)_2$; 0.04 mM ferric citrate; Allen trace elements (Allen, 1968); and 1% (w/v) Suc. The medium was buffered with 0.1 mM MES at pH 5.8 before autoclaving and was solidified via the addition of either 0.6% (w/v) agar or 0.25% (w/v) gellan gum (Caisson Laboratories). Seeds usually germinated within 3 to 4 weeks under a 16-h-light/8-h-dark cycle at 25°C. Seedlings were transferred to 1.5 cm tall petri plates containing solidified PMM for further growth. Plates were wrapped with Parafilm and grown at 25°C under a 16/8-h photoperiod at 50 μmol m^{-2} s^{-1}.

To induce stem gland development and establish the symbiosis, plants were transferred to N-free PMM [PMM in which KNO_3 is replaced with equimolar KCl and $Ca(NO_3)_2$ with equimolar $CaCl_2$]. Suc was also omitted from this medium. The pH was adjusted to 6.0 before autoclaving. Photographs of glands were taken using a Nikon Coolpix 995 digital camera mounted on top of an Olympus SZ-CTV stereo microscope.

Cultivation of *Nostoc punctiforme* and Induction of Hormogonia Differentiation

Nostoc punctiforme (ATCC 29133), obtained from J.C. Meeks (University of California at Davis), was maintained in liquid N-free BG11 medium (Rippka et al., 1979) and on N-free PMM medium solidified with 0.5% (w/v) gellan gum and grown under the same conditions as *G. manicata* plants.

For hormogonia induction, *N. punctiforme* taken from a 2- to 3-week-old N-free PMM plate-grown culture was streaked out onto a fresh N-free PMM plate. One milliliter of sterilized 0.5 mM MES buffer (pH 6.0) was placed on top of the cells to facilitate fragmentation of clumps. Nearly all the Nostoc filaments turned into hormogonia on the plate after overnight incubation at 25°C. Hormogonia were collected for inoculation by washing the plate with 1 to 2 mL of sterile 0.5 mM MES buffer (pH 6.0) and concentrated by transferring the liquid to a 1.5-mL centrifuge tube. After the hormogonia settled to the lower half of the tube, excess water was removed from the top to obtain 0.5 mL of a hormogonia-enriched solution. Photographs of *N. punctiforme* on solid medium were taken using a Nikon Coolpix 995 digital camera mounted on top of a Nikon Eclipse E200 microscope.

Inoculation of Stem Glands with Hormogonia

To establish the symbiosis, *G. manicata* seedlings were transferred to N-free PMM medium for the induction of gland development. The inoculation was performed on N-free plates when four to five red pigmented glands were visible on each seedling. A 10-μL droplet of the hormogonia solution was placed on top of each stem gland. Inoculated plants were maintained on the plates until signs of colonization were visible by eye. They were then transferred to polypropylene culture boxes (PhytoTechnology Laboratories) containing fresh N-free PMM medium for subsequent growth.

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