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Nostoc Talks Back: Temporal Patterns of Differential Gene Expression During Establishment of *Anthoceros-Nostoc* Symbiosis

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Abstract

Endosymbiotic associations between hornworts and nitrogen-fixing cyanobacteria form when the plant is limited for combined nitrogen (N). We generated RNA-seq data to examine temporal gene expression patterns during the culturing of N-starved *Anthoceros punctatus* in the absence and the presence of symbiotic cyanobacterium *Nostoc punctiforme*. In symbiont-free *A. punctatus* gametophytes, N starvation caused downregulation of chlorophyll content and chlorophyll fluorescence characteristics as well as transcription of photosynthesis-related genes. This downregulation was reversed in *A. punctatus* cocultured with *N. punctiforme*, corresponding to the provision by the symbiont of N_2-derived NH_4^+, which commenced within 5 days of coculture and reached a maximum by 14 days. We also observed transient increases in transcription of ammonium and nitrate transporters in a *N. punctiforme*–dependent manner as well as that of a SWEET transporter that was initially independent of N_2-derived NH_4^+. The temporal patterns of differential gene expression indicated that *N. punctiforme* transmits signals that impact gene expression to *A. punctatus* both prior to and after its provision of fixed N. This study is the first illustrating the temporal patterns of gene expression during establishment of an endosymbiotic nitrogen-fixing association in this monophyletic evolutionary lineage of land plants.

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A few lineages of land plants have an ability to establish symbiotic associations with nitrogen (N)-fixing bacteria, allowing them to colonize N-poor habitats (Mus et al. 2016). These plants share with others a set of common problems related to surviving N starvation, i.e., how to make more efficient use of scarce environmental nitrogen and where to put excess and potentially dangerous reductant that comes with the metabolic imbalance of excess photosynthetic capacity. Plants capable of establishing N_2-fixing associations must be able to attract such a bacterial partner and maintain an environment that is conductive to N_2 fixation, including a metabolic source of reductant. Since proteins of the nitrogenase enzyme complex are highly sensitive to oxygen (Gallon 1981), the plant partner must also protect the bacteria from even low levels of O_2. Legumes address the problem by providing their rhizobia partners with a form of hemoglobin that sets O_2 tension within a thin window (O'Brian 1996), low enough to avoid inactivation of nitrogenase but high enough to sustain the oxidative phosphorylation on which rhizobia rely (Gálvez et al. 2000).

Certain multicellular N_2-fixing cyanobacteria solve the O_2 problem differently, by forming, at periodic intervals, specialized micro-oxic cells called heterocysts, in which N_2 fixation can take place without risk of inactivation (Wolk et al. 1994). They, therefore, do not need the host plant for O_2 protection, and, perhaps consequently, heterocyst-forming cyanobacteria (mainly of the genus *Nostoc*) have established endosymbiotic associations with representatives of four divisions of land plants, namely, spore reproducing bryophytes (hornworts, two species of liverworts and perhaps peatmosses) and ferns (*Azolla* spp.) as well as seed-reproducing gymnosperms (cycads) and angiosperms (*Gunnera* spp.) (Adams 2000). A single strain, *Nostoc punctiforme*, can enter into productive associations with a broad range of partners (Meeks 1998). Because of this and because of the tools available to modify its genome (Cohen et al. 1994, 1998), *N. punctiforme* has become a model organism for the study of cyanobacterial cellular differentiation and plant-cyanobacterial associations (Meeks 2009; Meeks et al. 2002).

Anthoceros punctatus is the most highly studied plant host for cyanobacterial associations (Li et al. 2020). It is a bryophyte, one of two monophyletic lineages that make up land plants. Bryophytes (mosses, liverworts, and hornworts) lack vascular tissue, while the second lineage, tracheophytes (all other land plants), have characteristic lignin-containing vascular tissue (Szövényi et al. 2021). Bryophytes may be the oldest lineage of land plants to form stable, long-lasting endosymbiotic associations with nitrogen-fixing bacteria, in this case photoautotrophic and heterocyst-forming cyanobacteria (Sprent and Raven 1985; Warshan et al. 2018). Understanding how bryophytes establish

associations with cyanobacteria is, therefore, relevant to describing the evolution of an unusual symbiosis between photoautotrophic partners and may inform approaches to engineering cereal plants to form N_2-fixing symbioses (Mus et al. 2016; Oldroyd 2013; Pankievicz et al. 2019).

The initiation of association between *A. punctatus* and *N. punctiforme* shows several stages in which the plant influences cyanobacterial behavior. First, *A. punctatus*, when starved for nitrogen, produces one or more extracellular factors that induce the normally non-motile filaments of *Nostoc* spp. to differentiate into gliding filaments called hormogonia (Campbell and Meeks 1989). Hormogonium motility is essential but not singularly sufficient for establishment of functional cyanobacterial-plant associations (Meeks 2009). The hormogonium-inducing factor (HIF) from *A. punctatus* has yet to be identified. The only known factor with HIF activity was identified, from coralloid roots of *Cycas revoluta*, as a family of diacylglycerols (Hashidoko et al. 2019).

Hormogonium filaments lack heterocysts, do not fix N_2, and are growth-arrested (Tandeau de Marsac 1994). Hormogonia are transient; in laboratory culture, they return to the vegetative growth state within around 3 days after differentiation, with the filament terminal cells first differentiating into heterocysts, if cultured in the absence of combined N (Campbell and Meeks 1989; Meeks and Elhai 2002). *A. punctatus* may also release chemoattractants, by analogy with another bryophyte host and a variety of tracheophytes (Nilsson et al. 2006). Once hormogonia have colonized slime cavities on the ventral surface of the gametophyte, an *A. punctatus*–derived factor represses further hormogonium differentiation by activating transcription of a genetic locus in *N. punctiforme* with homology to hexuronic acid metabolism (Campbell et al. 2003; Cohen and Meeks 1997). Another genetic locus in *N. punctiforme* encodes a polyketide that acts as an autogenic repressor of hormogonium differentiation (Liaimer et al. 2015), which could also function in symbiosis. Under these conditions, infection is confined to the initial period of coculture, from 0.5 to less than 3 days, because growth for several weeks is necessary to dilute the repression factor (Meeks 1998).

Colonization of the slime cavity is followed by growth of associated *Nostoc* spp. and the differentiation of heterocysts. Here too, *A. punctatus* affects cyanobacterial behavior. Within the cavity, the frequency of heterocysts is about threefold higher than in the free-living state, and the rate of N_2-fixation is more than fourfold greater than in free-living *Nostoc* spp. Fixed nitrogen in the form of nitrate ordinarily suppresses heterocyst differentiation of both free-living and associated *Nostoc* spp., but a mutant *Nostoc* sp. strain PCC 9305 defective in nitrate assimilation is unresponsive to nitrate in the free-living state (producing heterocysts). The mutant differentiates heterocysts in association with *A. punctatus*, but symbiotic differentiation is repressed by nitrate (Campbell and Meeks 1992). Evidently, the N-starved plant exerts control over the initiation of heterocyst differentiation.

At the same time, assimilation of fixed N and phototrophic CO_2 fixation are depressed in *Nostoc* spp. associated with *A. punctatus* (Meeks 1998, 2003). Consequently, approximately 80 to 90% of the N_2 fixed by associated *Nostoc* spp. is released as ammonium to support growth of *A. punctatus*, with the remainder supporting slow growth of the symbiont (Meeks et al. 1985). In free-living *Nostoc* spp., heterocyst differentiation is induced only by growth in the absence of combined N and only when the strain shows physiological signs of N starvation, for example, by the breakdown of N-storage polymers cyanophycin and phycobiliproteins (Wolk et al. 1994). However, in *Nostoc* spp. associated with *A. punctatus*, these polymers remain intact even as heterocysts are differentiating (Meeks 1998; Meeks and Elhai 2002). The normal connection between N starvation and heterocyst differentiation appears to be subverted by *A. punctatus*. The decreased rate of photosynthetic CO_2 fixation indicates that a characteristic of endosymbiotic *Nostoc* spp. is heterotrophic growth. Hexoses such as sucrose, fructose, and glucose support dark nitrogen fixation (Steinberg and Meeks 1991) and dark growth (Summers et al. 1995) in *A. punctatus*. In epiphytic symbioses, trehalose has been implicated as a *Nostoc* sp. carbon source in peatmoss (*Sphagnum augustifolium*) (Carrell et al. 2021) and isotopically labeled organic carbon was demonstrated to be transferred to *N. punctiforme* in a feathermoss (*Pleurozium schreberi*) (Stuart et al. 2020).

The goal of this work was to set a framework for study of the signals that pass between *A. punctatus* and *Nostoc* spp. To do this, we have compared physiological signposts that occur during the establishment of the association with the expression of plant genes. Apart from a snapshot RNA-seq experiment reported along with the genome sequencing of the hornworts *A. punctatus* and two strains of *Anthoceros agrestis* (Li et al. 2020), nothing is known regarding genes involved in the infection of N-starved *Anthoceros* spp. or any other plant by *Nostoc* spp.

It must be noted that, by using the genetically tractable *N. punctiforme*, we acknowledge that the strain does not form a long-lasting association with *A. punctatus* compared with the original isolate *Nostoc* sp. strain PCC 9305, whose association can last for up to 3 months before it begins to outgrow *A. punctatus* in the laboratory (Enderlin and Meeks 1983). Unlike *Nostoc* sp. strain PCC 9305, *N. punctiforme* does not differentiate even a low level of hormogonia in the symbiotic cavity and, therefore, it cannot reinfect the margins of growing gametophyte tissue (E. L. Campbell and J. C. Meeks unpublished data). A second goal of this work is to establish whether results obtained with *N. punctiforme* over the first weeks of the association may be applicable to natural associations. Finally, a third goal is to explore the role of the common symbiotic signaling pathway (CSSP) in the *A. punctatus*-*N. punctiforme* symbiosis. The CSSP is composed of genes related to actinobacterial, rhizobial, and mycorrhizal colonization of plants, pertinent to nearly all land plants (Harris et al. 2020; Horváth et al. 2011; MacLean et al. 2017; Oldroyd 2013), but its role in plant-cyanobacterial associations is unknown.

To pursue these goals, we performed an analysis of the expression of *A. punctatus* genes over the course of N starvation, using gametophyte tissue incubated in the absence or the presence of *N. punctiforme* and looking for connections between the patterns of expression of specific genes and the physiological events that occur over the course of establishment of the plant-cyanobacterial association.

RESULTS AND DISCUSSION

Time course of *N. punctiforme* colonization of *A. punctatus*, induction of nitrogenase activity, and change in chlorophyll (Chl) content in N-starved gametophyte tissue.

Preliminary experiments were conducted to define morphological and physiological characteristics of *A. punctatus* during time courses of N starvation in the absence or the presence of *N. punctiforme*. *A. punctatus*, grown continuously in NH_4NO_3, was transferred to growth medium lacking a N source. The N-replete grown sample is the time 0 control for all experiments, with *N. punctiforme* added to one flask and the remaining flask containing only *A. punctatus*. Gametophyte samples were removed for assays of microscopically visible *Nostoc* colonies, acetylene reduction assays (diagnostic of nitrogenase activity), and to determine photosystem (PS) II quantum yield. Based on these results, subsequent experiments were conducted for extraction of total RNA and Chl at days 2, 5, 7, 10, 14, and 28 after initiation of coculture or continued N starvation.

The productive colonization of *N. punctiforme* within the slime cavities of *A. punctatus* gametophyte tissue does not happen immediately. It is the consequence of a multistep process requiring prior plant-dependent induction of hormogonium differentiation and chemoattraction of hormogonia (**Meeks 2003**, **2009**). *N. punctiforme* colonies were routinely visible in gametophytes under the microscope after 7 days of coculture and were only rarely observed earlier (Fig. 1A). The colonies were present mostly at the tip of the gametophytes, near the marginal meristem. The color of colonies observed on day 7 was relatively light, but they grew darker with time up to 14 days after coculture, reflecting growth of symbiotic *N. punctiforme* in the slime cavity. The space between the gametophyte margin and the colonies increased as the tissue continued to grow (Fig. 1A), but no new infections were observed over the 28-day period (normalized to fresh weight [FW] of gametophyte tissue). The reconstituted *A. punctatus*–*N. punctiforme* tissue retained its green color throughout the experimental time course. In contrast, when symbiont-free *A. punctatus* was N-starved, the gametophytes appeared pale green on day 2, then turned yellow to pale yellow as incubation time continued (Fig. 1B).

Fig. 1.
Morphological and physiological changes in symbiotic and symbiont-free gametophytes during N starvation. Micrographs of colonization of *Nostoc punctiforme* inside the slime cavities of **A,** *Anthoceros punctatus* gametophytes and **B,** symbiont-free *A. punctatus* gametophytes and their visual changes with time during the experimental time course. **C,** Average rates (±standard deviation [SD]) of acetylene reduction (nitrogenase activity) from symbiotic gametophytes. **D,** Average content (±SD) of total chlorophyll and **E,** dark-adapted (20-min darkening) maximum quantum yield of photosystem II, estimated by chlorophyll fluorescence (F_v/F_m) in the gametophytes of symbiotic and symbiont-free *A. punctatus* under N-starvation conditions.

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Acetylene reduction to ethylene was observed within 5 days of coculture, before *N. punctiforme* colonies were routinely visible under the microscope. The rate of acetylene reduction by symbiotic gametophytes increased with time from 0.109 nmol per minute per gram of FW on day 5 to 11.423 nmol per minute per gram of FW on day 14 before declining to 12% of the maximal rate by day 28 ($P < 0.001$) (Fig. 1C). A linear regression analysis of the data from days 5 to 14 yielded an intersection on the *x* axis for the initiation of N_2 fixation between days 2 and 3 (data not shown). The inability of *N. punctiforme* to form long-lasting associations with *A. punctatus* may explain, in part, the decline in acetylene reduction between days 14 and 28 after the initiation of coculture. The symbiotic *N. punctiforme* day 14 rate of acetylene reduction is similar to the steady state rate of *A. punctatus* colonized with *Nostoc* sp. strain PCC 9305 (also known as UCD 7801) (**Steinberg and Meeks 1991**), isolated from *A. punctatus*.

N limitation in plants impairs the photosynthetic machinery, causing significant damage in the reaction centers of PS II, leading to a major reduction in variable Chl *a* fluorescence (F_v/F_m) (**Zhao et al. 2017**). **Murchie and Lawson (2013)** proposed that a F_v/F_m value of <0.6 be accepted as a screening technique of PS II to indicate stress conditions in plants. Such a response was observed during N limitation of *A. punctatus* (Fig. 1E). The maximum dark-adapted PS II quantum yield was estimated by in vivo Chl fluorescence (F_v/F_m). A sustained F_v/F_m value ranging from 0.729 to 0.819 was observed in symbiotic *A. punctatus* gametophytes. N starvation resulted in a decrease in F_v/F_m of symbiont-free *A. punctatus* from 0.819 at day 0 to 0.574 (stressed phase) by day 28 ($P < 0.001$).

Total Chl content of N-starved cocultured and symbiont-free *A. punctatus* was estimated by calculating the Chl *a* and Chl *b* content from gametophyte tissues. The total Chl content in symbiotic gametophytes varied with time from 5.23 to 3.3 µg per 10 mg of FW, whereas the total Chl content declined from 5.23 to 0.79 µg per 10 mg of FW in symbiont-free *A. punctatus* ($P < 0.001$) (Fig. 1D). The decline in Chl content during N starvation of symbiont-free *A. punctatus* resembled the response by most algal and plant species (**Nunes-Nesi et al. 2010**; **Schmollinger et al. 2014**).

Improved genome assembly and annotation of *A. punctatus*.

The raw sequence data from *A. punctatus* used by **Li et al. (2020)** to assemble a genome of 132.8 Mb was reprocessed as described below and led to a new assembly of 134.6 Mb. The new assembly has substantially greater contiguity, as judged by a doubling of the contig N_{50} from 1.7 to 3.3 Mb. The number of predicted gene models from the new assembly decreased from 25,426 to 23,021 (Supplementary Dataset S1), but there was a considerable increase, from 85 to 92%, in complete genes identified by BUSCO v5.1.2, using the database Viridiplantae ODB10. Of all the predicted genes, 69% could be functionally annotated (Supplementary Dataset S2).

Global RNA-seq analysis.

Principal component analysis (PCA) was used to assess variation in gene expression values among the three biological replicates. The plot of the 39 points (three replicates, six timepoints, two culturing conditions, plus 1 common time zero) on the two PC axes showed that the replicates of 12 of the 13 conditions clustered together (Supplementary Fig. S1). The outlier was the T0 (time zero) point, where one of the replicates was distant from the others. These results justified the use of the replicates in determining significant differences in gene expression.

Identification of differentially expressed genes (DEGs) common to symbiotic and symbiont-free *A. punctatus*.

A. punctatus was found to have 1,448 genes with statistically significantly different time courses of expression ($P < 0.05$), comparing expression with or without *N. punctiforme* to time 0. The total was almost evenly split between higher expression in the presence of *N. punctiforme* (44%) or lower expression in its absence (56%). The identities of 1,210 genes (84%) with functional annotations were predicted via eggNOG (Supplementary Dataset S3).

The DEGs were manually organized into the 10 major metabolic groupings shown in Supplementary Figure S2 to allow analysis of their expression patterns in the context of cellular growth and its regulation. The most highly represented genes are present in the unassigned function and core metabolism categories, which might be expected in the global transcriptomic analysis of a growth stress response. The complete functional analysis is presented in Supplementary Dataset S3 with a gene list, more detailed functional categories, and normalized expression values. Supplementary Note S1 offers a guide to the functional groups.

Cluster analysis of the DEGs.

The DEGs were subjected to cluster analysis, which assigns genes to a pre-specified number of clusters (nine) according to common expression patterns of the genes over the time course. The expression patterns of the nine clusters are plotted in Figure 2. Each panel reflects the transcriptional patterns of the same genes of *A. punctatus* during combined-N starvation in the absence (symbiont-free) and the presence (symbiotic) of *N. punctiforme* (Supplementary Dataset S3 provides the identity of the genes in each cluster).

Fig. 2.
Temporal expression profiles for differentially expressed genes from cocultured and symbiont-free *Anthoceros punctatus* gametophytes. The number of clusters was user selected and then arbitrarily numbered by the algorithm. The ordinate is the median log of the variance stabilizing transformation–normalized gene expression for that set of genes ($P \leq 0.05$). The abscissa is time in days since initiation of N starvation and addition of *Nostoc punctiforme* (in the case of the cocultured samples). Orange and green lines indicate differential gene expression profiles of symbiont-free and cocultured gametophytes, respectively.

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The nine clusters may be combined into two groups, according to the influence of *N. punctiforme* on the pattern of gene expression. The first group, clusters 4, 5, 8, and 9, showed essentially the same patterns in the absence or the presence of *N. punctiforme*. The shape of the plot of the symbiotic *A. punctatus* in cluster 5 resembles that of two clusters in the second group, clusters 3 and 7, namely, a sharp downregulation followed by a 5-to-10-day lag and then upregulation before peaking at 14 days of incubation. This appears to reflect a hybrid response, because there was slower upregulation in the absence of *N. punctiforme* and the level of expression in both tissues approached the time 0 value by day 28. Similarly with the other three clusters in the group, the transcriptional patterns appeared to be influenced by but not dependent on *N. punctiforme*.

Most interesting is the second group composed of clusters 1, 2, 3, 6, and 7. Relative to *A. punctatus* alone, the presence of *N. punctiforme* unequivocally either repressed the N starvation–induced upregulation of transcript accumulation (clusters 1 and 6) or enhanced the transcription accumulation of genes that were downregulated in its absence or, in some cases, its presence (clusters 2, 3, and 7). However, beyond simply up- or downregulation, there are significant temporal differences in the responses. For examples, in cluster 6, the repressive effect of *N. punctiforme* appeared to be immediate, whereas, in cluster 1, N starvation elicited transcriptional upregulation in both cultures, which, in the symbiotic culture, was followed by a short period of stable expression and then a first order decline to the time 0 level. In cluster 2, the symbiotic tissue showed an immediate upregulation in transcripts, which remained elevated until at least day 10, before beginning a decline to slightly less than the time 0 value, while that in symbiont-free *A. punctatus* stayed constant through day 2 before they slowly declined to a lower level. The transcriptional patterns in clusters 3 and 7 of group 2 differed in the timing of the enhanced accumulation. The transcripts of genes in cluster 3 began to increase accumulation from the repressed level after 5 days of incubation, while, in cluster 7, the increase started after day 10. In both cases, the level of accumulation returned to at or near the time 0 level, which may indicate return to a steady state.

Three clusters of group 2 also can be divided with respect to the onset of N_2 fixation. The symbiotic temporal patterns in clusters 1, 3, and 7 strongly correlated with the pattern of the onset of symbiotic nitrogenase activity and accounted for 42% of the DEGs. Conversely, the symbiotic temporal patterns in clusters 2 and 6 were initiated prior to and, initially, appeared independent of symbiotic N_2 fixation, although the persistence of their transcription pattern might have been dependent upon N_2-derived NH_4^+; these clusters accounted for 25% of the DEGs.

We suggest that the two different overarching patterns in this second group of clusters have implications regarding the nature of symbiotic interactions between *A. punctatus* and *N. punctiforme*. This will become apparent in the sections that follow, in which different physiological consequences of N starvation on photoautotrophic growth of *A. punctatus* are related to the transcriptional responses to coculture with *N. punctiforme* of specific genes within the clusters.

Genes related to the metabolic instability caused by N starvation.

One consequence of N starvation is that reductant, previously used to synthesize central metabolism precursors and monomers, now accumulates, which in oxygenic photosynthetic organisms can lead to the generation of toxic reactive oxygen species (**Foyer and Shigeoka 2011**). One can hypothesize three different strategies by which the plant could respond to this stress: i) decrease the rate of photosynthetic reductant generation (**Logan et al. 1999**; **Paul and Driscoll 1997**), ii) increase the uptake of low levels of an existing source or acquisition of an alternative source of combined N (**Crawford and Glass 1998**; **Glass et al. 2002**; **Liu et al. 2017**), or iii) utilize an organic reductant sink whose metabolic products can be stored or excreted (**Hsieh et al. 2018**). We will consider these alternatives below.

Genes related to photosynthetic characteristics.

The physiological measurements indicated that N starvation of symbiont-free *A. punctatus* resulted in decreased Chl content and declining PS II quantum yield, which are typical responses in algae and plants (**Sayed 1998**). It may be informative to compare the timing of these responses to the transcription of nuclear-encoded, chloroplast-localized proteins and complexes associated with photosynthetic reaction centers, electron transport, and CO_2 fixation.

There were 33 DEGs encoding proteins indirectly or directly related to photosynthesis (Supplementary Dataset S3). Sixty percent of the genes are present in cluster 3, which contains genes that were initially downregulated and then upregulated in the presence of *N. punctiforme* in a temporal pattern correlated with the production of N_2-derived NH_4^+. Figure 3 shows the time courses of genes encoding one of ten proteins related to light harvesting complexes (LHC) (Fig. 3A), one of two oxygen-evolving enhancer (Fig. 3C), a PS I reaction center protein and probable ferredoxin docking protein (Fig. 3D), and the ribulose bisphosphate carboxylase/oxygenase (RuBisCO) small subunit (Fig. 3E). The rapid transcriptional downregulation of these core photosynthetic genes appeared to be uncoupled from the slower decline in total Chl content and even slower decline in PS II quantum yield.

Fig. 3.
Temporal patterns of normalized expression of genes encoding proteins involved in photosynthesis. The ordinate is the mean (±standard deviation) normalized gene expression ($P < 0.05$). The differentially expressed genes encode proteins for **A,** a representative light harvesting complex, **B,** enhanced O_2 evolution, **C,** photosystem (PS) II reaction centers, **D,** PS I reaction centers, **E,** RuBisCO small subunit, and **F,** xanthophyll metabolism from cocultured (solid lines) and symbiont-free (broken lines) *Anthoceros punctatus* gametophytes. Time is in days.

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Some of these internal differences might be due to the relatively low light intensity (approximately 65 µmol photons per square meter per second) used during culture, which could slow bleaching and photooxidation. This suggestion is supported, in part, by the observation that a gene encoding a protein involved in xanthophyll metabolism is present in cluster 7, where it showed rapid downregulation in symbiont-free and symbiotic tissues and was subsequently upregulated in symbiotic tissue between days 14 and 28 to approximately the time 0 level (Fig. 3F). This pattern implies a degree of N-starvation response, although it was the reverse of that anticipated in protection from a highly reduced photosynthetic electron transport system. Xanthophylls are present in association with Chls in the LHCs, where they operate in a cycle of oxidation and reduction to quench excitation generated by high light intensities (**Latowski et al. 2011**; **Niyogi et al. 1997**). It is of interest to note that upregulated transcription of photosynthetic and other DEGs in cluster 3 appeared to respond to lower concentrations of N_2-derived NH_4^+ (submaximal rate of nitrogenase) than DEGs in, for example, cluster 7. This implies a preferential transcription and translation of genes defining the trophic level of the plant and more specifically that reaction center stabilization and RuBisCO assembly were prioritized over high light protection.

Many genes encoding core components of the PS reaction centers and of electron transport chains as well as the large subunit of RuBisCO are localized in the plastid genome (**Allen 2015**). They could also be differentially transcribed, and indeed, their transcription has been proposed to be subject to redox control (Allen 2015). However, they are not in our dataset, which was confined to genes with poly-A extensions (and therefore nuclear origin).

We conclude that a major physiological and transcriptional response to N starvation by *A. punctatus* was to lower the photosynthetic potential, perhaps largely by destabilizing the PS II reaction center complex and limiting electron transfer out of the PS I complex. The lowered potential was subsequently reversed by symbiotic association with N_2-fixing *N. punctiforme*.

Genes related to N acquisition.

Many of the genes encoding proteins for N acquisition and assimilation in *A. punctatus* occur in multigene families (Supplementary Table S1). Some N-starved plants induce the transcription of either transport systems for ammonium (AMT), nitrate/nitrite (NTR), or both (Calabrese et al. 2017; Krapp et al. 2011). Redundancy in AMT genes is common in land plants, (Couturier et al. 2007; Loqué and von Wirén 2004). The genes have distinct phylogenies that can be subdivided into subfamilies and clades and, in tracheophytes, gene products and isozymes are often localized in specific tissues and organs, which assist in understanding the physiological roles of gene family members. Those bryophytes that establish endosymbiotic associations may also have tissue differentiated from the bulk gametophyte thallus, such as sporophytes, rhizoids, and slime cavity cells, which, in the liverwort *Blasia pusilla*, elaborate septate, branched filaments of metabolite transfer cells that increase in profusion after *Nostoc* sp. colonization (Rodgers and Stewart 1977), and in which isozymes could be localized. However, the *A. punctatus* gametophytes lacked attached sporophytes and we did not attempt to dissect rhizoids or slime cavity cells here.

The bryophyte liverwort *Marchantia polymorpha*, has five AMTs in the AMT1 clade (McDonald and Ward 2016). The *A. punctatus* genome contains seven genes annotated as AMTs, four of which were expressed under the growth conditions considered here (Supplementary Table S1). Of the four expressed *A. punctatus* AMT genes, one (g54810) was classified as a DEG (cluster 1) and encodes a version of AMT1;5 that was upregulated early in N starvation and remained high in the absence of *N. punctiforme* but declined in its presence (Fig. 4A). This temporal pattern can be understood as the plant searching for exogenous ammonium, satisfied when N_2-derived NH_4^+ became available. The other three AMT genes were not DEGs and were expressed at levels ranging from 70 to 1% of that of g54810. Two of the latter genes, g162610 and g187510, encode proteins that resemble an AMTB-like transporter from rhesus monkey, while the third, g215080, encodes an unusual hybrid protein with AMT and protein phosphatase domains. Experiments with fluorescently tagged proteins could determine whether the AMTs are localized in specific tissues.

Fig. 4.
Temporal patterns of normalized expression for genes involved in N acquisition and assimilation. The ordinate is the mean (±standard deviation) normalized gene expression ($P < 0.05$). The selected genes encode **A,** an ammonium transporter, **B,** a nitrate transporter (NRT 2.2), **C,** a putative nitrate transporter (NPF 8.1), **D,** signal transduction P_{II}, **E,** a plastid-localized glutamine synthetase (GS2), **F,** a cytosolic glutamine synthetase (GS1;1), **G,** the complete ferredoxin glutamate synthase, and **H,** the expressed NADH glutamate synthase from cocultured (solid lines) and symbiont-free (broken lines) gametophytes. Time is in days.

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There are two genes putatively encoding high affinity nitrate transporters in the NTR2.2 family (g128250 and g119600). Both genes were highly expressed (Supplementary Table S1). Neither gene was flagged as a DEG (Supplementary Dataset S3), however, both appeared to be expressed in the pattern of cluster 1 (Fig. 4B, g119600). These transcripts did not pass through the false discovery rate filtering nor were they statistically significant at $P \leq 0.05$ when identifying the differential expression profiles, using maSigPro. However, when the high affinity nitrate transporter NRT2.2 data were separately statistically analyzed using Tukey's honestly significant difference (HSD) in R, we observed significant changes in cocultured *A. punctatus* gametophytes (at days 14 and 28 in comparison with day 0). The protein encoded by g119600 strongly resembles reference NTR proteins throughout its length, but the protein encoded by the other NTR2.2 member (g128250) is much less similar and lacks transmembrane domains. Its actual function is, therefore, not clear. In summary, *A. punctatus* appears to possess at least one fully functional high affinity nitrate transporter and its expression pattern was consistent with an attempt to increase nitrate uptake, an effort that was abandoned when N_2-derived NH_4^+ became available.

The transcriptome also contains 32 genes encoding proteins of one or both the low or variable affinity nitrate and proton-dependent oligopeptide transporters in the NTR1.1 PTR family (Sun and Zheng 2015), now referred to as NPF (Wang et al. 2018) (Supplementary Table S1). Four of the genes were identified as DEGs and assigned to clusters 2 (two genes), 6, and 7. The gene in cluster 6 (g138870) showed early upregulation in the absence of *N. punctiforme* and its upregulation was repressed in symbiotic tissue well before provision of N_2-derived NH_4^+ (Fig. 4C). However, we hesitate to draw conclusions from these results due to the ambiguity of the physiological role of this protein family.

It has been documented that plants can use a variety of exogenous amino acids and even proteins as N sources for growth (**Mertz et al. 2019**; **Miller et al. 2008**; **Paungfoo-Lonhienne et al. 2008**). In the peatmoss epiphytic association, amino acids (arginine, aspartate, glutamine, and methionine) and purines (cytidine and xanthosine) were reported to be supplied by the *Nostoc* sp. symbiont (**Carrell et al. 2021**). Moreover, endosymbiotic *Nostoc* spp. were reported to supply amino acids to cycads (**Pate et al. 1988**). Amongst the DEGs that are annotated as amino acid and other organic N transporters, the gene with the highest level of expression (g184100, cluster 7) was a putative lysine and histidine transporter. Its expression pattern in response to N starvation, repression followed by recovery in the presence of *N. punctiforme*, is inconsistent with a role in scavenging nitrogen. It remains possible that the symbiont may export lysine or histidine and induce transcription of the transporter in *Anthoceros* spp., but we have no evidence for such a possibility. Taken together, the patterns of transcription support a role for ammonium and nitrate transport but not organic N in the response of *A. punctatus* to N starvation.

Genes related to N assimilation.

In general, N starvation also induces upregulation of N signaling and assimilatory proteins in bacteria and plants. Many eubacteria, all cyanobacteria, and plants assimilate ammonium, derived from N_2, nitrate, or urea, by the glutamine synthetase (GS) and glutamate synthase (GOGAT, an acronym for glutamine: 2-oxoglutarate amido transferase) pathway. This pathway has been shown to be operative in vivo by symbiotic and symbiont-free *A. punctatus* (**Meeks et al. 1983**, **1985**).

N assimilation is regulated in plants by the N–signal transduction protein P_{II}, which is localized in chloroplasts and encoded by the nuclear gene *GLB1*. The state of P_{II} serves to represent in various cells the ratio of carbon (2-oxoglutarate; 2-OG) to either nitrogen (glutamine or glutamate), the energy charge (ATP/AMP + ADP), or both. In Eubacteria, especially the Proteobacteria, reversible uridylylation of P_{II} results in modulation of ammonium transport (AmtB) and GS expression and activity (**Merrick 2015**). In cyanobacteria and plants, P_{II} reversibly binds 2-OG (unicellular cyanobacteria), glutamine (green algae and plants), and ATP or ADP in a competitive manner. The ligand-bound P_{II} associates with the key enzyme of arginine synthesis, *N*-acetyl-L-glutamate (NAG) kinase, reducing arginine feedback inhibition of the enzyme and enhancing synthesis of NAG phosphate (**Selim et al. 2020**). Thus, in the presence of high concentrations of ammonium, ligand-bound P_{II} protects the glutamate pool in Proteobacteria and converts glutamate into an arginine N-storage state in cyanobacteria and plants. In *Arabidopsis thaliana*, *GLB1* is transcriptionally upregulated by light and sucrose and is downregulated in the dark and in the presence of asparagine, glutamine, and glutamate (**Hsieh et al. 1998**). Conversely, in the green alga *Chlamydomonas reinhardtii*, *GLB1* is upregulated in the absence of ammonium (**Zalutskaya et al. 2018**).

In symbiont-free and symbiotic *A. punctatus*, *GLB1* was upregulated about threefold during N starvation, with stable accumulation between days 7 and 10, prior to declining after day 10 (Fig. 4D). The fact that the temporal differential expression of *GLB1* was not materially altered by coculture with *N. punctiforme* and the resulting N_2-derived NH_4^+ implies that P_{II} may be involved in more than N stress (**Chellamuthu et al. 2013**).

In bacteria and some plants, limitation for ammonium results in upregulation of GS activity and synthesis. In algae and plants, there are two designated forms of GS proteins encoded by nuclear genes, distinguished by their molecular mass; GS1 is localized in the cytosol and its isozymes are distributed in various tissues and organs, whereas higher molecular mass GS2 is targeted to the plastids (**Swarbreck et al. 2011**). The plastid protein is typically encoded by a single nuclear gene, while three to five genes encode the GS1 cytoplasmic isozymes (**Swarbreck et al. 2011**).

In *A. punctatus*, eight nuclear genes are annotated as members of the GS (*glnA*) superfamily (Supplementary Table S1). The lone member identified as a DEG is g158060, encoding a GS2-type of protein with a signal peptide. As a representative of cluster 3, its transcripts initially declined in symbiont-free and cocultured *A. punctatus* before they increased to a maximum by 14 days of incubation in symbiotic tissue and then again declined (Fig 4E). The pattern implies that the initial decrease in transcription was in response to the absence of combined N and the subsequent increase in transcription was in response to the presence of N_2-derived NH_4^+. We speculate the pattern could be associated with the physiological status of the chloroplasts and variable photorespiratory flux. The much more highly expressed non-DEG g128160 encodes a GS1-type protein with no signal peptide and a defined cytosolic domain (Fig. 4F). Based on its expression levels (an order of magnitude higher than any other GS), cytosolic GS1;1 is the dominant GS assimilatory protein in the entirety of the

gametophyte tissue and its transcription is not under stringent N-control. It is of interest to note that two other genes (g141120 and g103610) encode GS proteins with signal peptides and defined non-cytoplasmic domains, implying targeting to unknown compartments. The latter is a fusion protein with an additional metallo(amido)hydrolase non-cytoplasmic domain (Supplementary Table S1).

GOGAT comes in two types, ferredoxin (Fd)-dependent GOGAT (*GLSF*; also known as *GLU1*) and NADH-dependent GOGAT (*GLTB*), both are encoded in the plant nucleus but active in plastids (**Suzuki and Knaff 2005**). In tracheophytes, enhanced transcription of GOGAT-encoding genes is tissue-specific in response to photosynthate and N metabolites (**Suzuki and Knaff 2005**). The genes encoding Fd-GOGAT are upregulated by light (especially red), sucrose, nitrate, ammonium, and other substrates and products of the GS-GOGAT pathway (**Suzuki and Knaff 2005**). Conversely, the NADH-GOGAT-encoding genes appears to be primarily under nitrate and ammonium control with the most robust expression in roots (**Suzuki and Knaff 2005**).

In contrast, regulation of GOGAT by N appears to be weak in *A. punctatus*, which has two genes for each of the two GOGAT types, none classified as DEGs. The Fd-GOGAT–encoding genes (g70690 and g70700) are present in tandem in the genome. The expression of both genes followed a time course similar to that of GS g128160 (Fig. 4G, showing g70690), but the transcripts of g70700 accumulated at about a 10-fold lower level than g70690. The lower-expressing protein encodes a signal peptide and an amidotransferase domain but lacks a normally present glutamate synthase domain (Supplementary Table S1). It is unclear whether the g70700 protein functions together with the g70690 protein or at all. The gene g63560 encodes a NADH-GOGAT that consists of the core *GLSF*-encoded protein's catalytic domains plus a mannosyltransferase domain and a pyridine nucleotide disulfide oxidoreductase domain in the N-terminal and C-terminal regions, respectively (Supplementary Table S1). This NADH-GOGAT was transcribed at a constant level (Fig. 4H), approximately two to four times lower than the g70690 encoded Fd-GOGAT. The second gene potentially encoding NADH-GOGAT showed no expression. Thus, one dominant FD-dependent GOGAT chloroplast protein and one NADH-dependent GOGAT cytosolic protein were present in *A. punctatus* under our experimental conditions.

Collectively, these results provide support for N control over key enzymes of ammonium and nitrate transport and, possibly, N–starvation associated assimilation in the case of a plastid GS2.

Genes related to potential sinks for excess reductant accumulation.

Candidates for organic end products that function as internally stored or excreted reductant sinks during N starvation include products such as polysaccharides, fatty acids and lipids, and secondary metabolites. We expected that the expression patterns for genes associated with such sinks should follow those shown in cluster 1, as depicted by the ammonium and nitrate transporters (Fig. 4).

The symbiotic cavity in gametophyte tissue contains considerable slime; therefore, secretory polysaccharide synthesis must happen, and upregulation of the pathway would be a relatively benign solution to the consequences of N starvation. However, neither the slime composition nor its biosynthetic pathway is known nor whether symbiotic *Nostoc* spp. contributes to its production. One glycosyltransferase-encoding gene (g23860), possibly involved in polysaccharide synthesis, is present in cluster 1 (Fig. 5A). Upregulated transcription of g23860 increased in the presence and the absence of *N. punctiforme* until day 5, when it began to decline in symbiotic *A. punctatus*; in symbiont-free *A. punctatus* transcript accumulation continued until it was more than double the time 0 value by day 14. While this reflects the pattern predicted of a reductant sink, its level of transcription appeared to be marginal for a sole sink.

Fig. 5.
Temporal patterns of normalized expression for genes involved in potential reductant sinks for photosynthetically generated reductant. The ordinate is the mean (±standard deviation) normalized gene expression ($P < 0.05$). The selected genes encode **A,** glycosyltransferase, **B,** a cytochrome P450, and **C,** terpene synthase from cocultured (solid lines) and symbiont-free (broken lines) gametophytes. Time is in days.

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Triacylglycerol (TAG) and lipid accumulation have long been known to occur in microalgae during N starvation (**Spoehr and Milner 1949**), even to the extent of serving as a possible source of biofuel (**Goncalves et al. 2016**). Lipid accumulation has been noted, as well, in seeds of certain plants, such as castor bean (**Chen et al. 2018a**). The genome of *A. punctatus* contains six genes encoding acyl carrier protein (ACP) synthases, involved in both fatty acid and TAG synthesis. Three ACP synthases were expressed: g14190, g129680, and g27940. Only g14190, identified as encoding a Kas1family protein, was a DEG in cluster 3, which is inconsistent with a predicted reductant sink. The two others were non-DEGs, and the remainder were not expressed. We detected the gene (g23940) encoding acyl-CoA:diacylglycerol acyltransferase (diacylglycerol-*O*-acyltransferase), converting 1,2-diacyl-*sn*-glycerol to TAG in the *A. punctatus* genome; it was moderately expressed as a non-DEG. Based on transcriptional characteristics relative to other plants and algae, fatty acid, TAG, and lipid accumulation hint at but do not appear to be sufficiently robust during N starvation of *A. punctatus* to unequivocally function as sole reductant sinks. These possibilities will need to be addressed biochemically.

Cytochrome P450 is a monooxygenase that consumes NADPH and whose activities are involved in numerous metabolic pathways, especially those leading to stress adaptation, development, and synthesis of secondary metabolites (**Xu et al. 2015**). Forty-six genes encoding cytochrome P450 were classified as DEGs, 83% are present in clusters 1 and 6. An example of the temporal expression pattern of one such gene (g134660) is presented in Figure 5B, in which initial upregulation in the absence and presence of *N. punctiforme* is shown, followed by repression in the presence of the symbiont. This latter observation, with the downward turn coinciding with the induction of symbiotic nitrogenase activity, is consistent with an enzyme that is involved in alleviating N stress through a reductant sink. It should be noted that an equally large number of genes encoding cytochrome P450 are grouped in cluster 6 (e.g., g57450), in which the presence of *N. punctiforme* repressed transcription before nitrogenase activity had been induced (Supplementary Dataset S3). These results indicate that specific cytochrome P450 proteins were well-expressed under N stress.

Terpenes are fundamental substrates for the synthesis of a variety of stable and volatile secondary plant products synthesized following a variety of abiotic and biotic stresses (**Chatterjee et al. 2017**, **2018**, **2020**). They could well function as reductant sinks. Of the 21 genes annotated as part of the terpene synthase family, two were classified as DEGs, both in cluster 6. The pattern of expression of one (g60250) is shown in Figure 5C. The presence of *N. punctiforme* completely suppressed the upregulation of transcription from the level present at time zero. This pattern could be consistent with a terpene as a reductant sink, whose synthesis was suppressed in the presence of *N. punctiforme*. However, we conclude the evidence is currently equivocal for the substantial accumulation of reduced secondary metabolites during N starvation in *A. punctatus*.

Collectively, these data indicate the response of *A. punctatus* to N starvation and its complementation by *N. punctiforme* appeared to be strongly involved in depression of photosynthetically generated reductant, a search for low concentrations of ammonium and nitrate in the environment, and not a single highly expressed pathway but, at best, a variety of low-level sinks for the photosynthetically generated reductant.

Genes related to symbiotic interactions.

Based on morphological, physiological, and biochemical data, we previously suggested that the signaling between hornwort and cyanobacterium is primarily unidirectional from plant to symbiont, apart from plant responses to N_2-derived NH_4^+ (**Meeks 1998**, **2009**). The results presented here call for a reconsideration of this view. The temporal patterns of clusters 1, 3, and 7 in symbiotic relative to symbiont-free gametophytes occur after the onset of N_2 fixation and can be explained as a response to N_2-derived NH_4^+, but the patterns of clusters 2 and 6 indicate an additional layer of signal transduction. The conspicuous upregulation of DEGs in cluster 2 and downregulation of DEGs in cluster 6 were apparent by day 2 of coculture, prior to the onset of N_2 fixation. We do not yet know whether the early signaling that occurs during the process of colonization is chemical in nature or requires physical interaction between the symbionts. Experiments to clarify the matter are in progress, using existing *N. punctiforme* mutants that differentiate hormogonia unable to infect gametophyte tissue and mutants that are infective but unable to fix N_2.

A central question is whether proteins that lie at the foundation of plant-arbuscular mycorrhizal associations, the CSSP, are also involved in the establishment of hornwort-cyanobacteria N_2-fixing symbioses. Nine genes encoding proteins of the CSSP (**Debellé 2020**; **Delaux and Schornack 2021**; **MacLean et al. 2017**; **Oldroyd 2013**; **Sellstedt and Richau 2013**) were reported to be present in the *A. punctatus* genome (**Li et al. 2020**). None of those genes were classified as DEGs, but some showed apparent differences in expression between symbiotic and symbiont-free conditions (Fig. 6). Therefore, the expression patterns of some of these genes were analyzed in the previous manner of the high-affinity nitrate transporter. In particular, STR2 (ABC transporter) (Fig. 6D) showed the pattern of DEG cluster 7 (the normalized expression at days 2, 5, 7, 10, and 14 was statistically significantly different from day 0 in symbiont-free gametophytes), while one copy of RAM1 (GRAS family transcription factor) (Fig. 6I) (the normalized expression at day 2, 5, 14, and 28 was statistically significantly different from day 0 in symbiont-free gametophytes) and low-expressing RAD1 (GRAS family transcription factor) (Fig. 6H) of cluster 1. The remaining genes encoding CSSP protein showed expression patterns that were not markedly altered by the presence of *N. punctiforme*: Castor (calcium channel) (Fig. 6A), Cyclops (calcium calmodulin-dependent binding to CCamK) (Fig. 6B), STR1 (ABC transporter) (Fig. 6C), SymRK (receptor-mediated signaling) (Fig. 6E), CCamK (calcium and calmodulin-dependent serine/threonine protein kinase) (Fig. 6F), and Vapyrin (protein kinase) (Fig. 6G). A LysM receptor kinase is involved in perception of nodulation factors (**Buendia et al. 2018**), and a gene (g02670) putatively encoding such a protein was present in the transcriptome at a high expression level (Fig. 6J). These results indicate that the sensing and immediate signaling capacities of the CSSP are present in gametophyte tissue to recognize a compatible symbiont, which could be *Nostoc* spp. or mycorrhizal fungi, or both; hornworts do form mycorrhizal associations (**Desirò et al. 2013**). Because the genes were constitutively transcribed at a high level in *A. punctatus*, involvement of the CSSP in cyanobacterial symbiosis with hornworts needs to be studied by mutational analysis, which has been reported achievable in *A. agrestis* (**Frangedakis et al. 2021**).

Fig. 6.
Temporal patterns of normalized expression for genes involved in symbiotic interactions. The ordinate is mean (±standard deviation) of normalized expression of genes encoding proteins **A,** Castor, **B,** Cyclops, **C,** STR1, **D,** STR2, **E,** SymRK, **F,** CCamK, **G,** Vapyrin, **H,** RAD1, **I,** RAM1, **J,** LysM receptor like kinase, and **K,** SWEET transporter from cocultured (solid lines) and symbiont-free (broken lines) *Anthoceros punctatus* gametophytes. Time is in days. Expression values for all genes except LysM (J) and SWEET (K) were obtained from the transcriptome. LysM and SWEET sorted into the differential transcriptome, whereas STR2 and RAM1 did not. These latter genes were analyzed by Tukey's honestly significant difference.

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In the epiphytic *Nostoc* spp.–moss associations, a novel observation has been that organic sulfur produced by the plant partner appears to be translocated to the *Nostoc* spp.–symbiont, and choline-*O*-sulfate, taurine, and sulfoacetate by chemical identity in peatmoss (**Carrell et al. 2021**), or alkanesulfonate by bioinformatics and *N*. *punctiforme* mutant analysis in feathermoss (**Stuart et al. 2020**). The three genes for taurine synthesis from cysteine or those for synthesis of nonspecific alkanesulfonates were not found in the *A*. *punctatus* eggNOG-annotated genome (Supplementary Dataset S2). Thus, we currently have no conclusive evidence whether *A*. *punctatus* makes organic sulfur available to its *Nostoc* spp. symbionts.

Transcription of gene g119980 encoding a sugar transporter (SWEET) in *A. punctatus* was previously identified as specifically upregulated in the presence of *N*. *punctiforme* (**Li et al. 2020**). The time course shown in Figure 6K shows that the activation of transcription of SWEET occurred early in the presence of *N*. *punctiforme*, prior to its provision of N_2-derived NH_4^+, implying that N starvation was not sufficient for induction of the gene and that something associated with *N. punctiforme* besides its ability to fix N_2 is critical. The time course of SWEET expression fits well with the hypothesis that *A*. *punctatus* provides sugars, such as sucrose, fructose, and glucose, that are known to support essentially the same or, under some conditions, a greater rate of symbiotic N_2-fixation by *Nostoc* spp. in association with *A*. *punctatus* (**Steinberg 1990**; **Steinberg and Meeks 1991**). In addition, we have observed a dependence on a *N*. *punctiforme* sugar permease for development of a functional symbiotic association (**Ekman et al. 2013**).

Conclusions.

We have described an association between the bryophyte *A*. *punctatus* and the cyanobacterium *N*. *punctiforme* that permits an investigation into the time course of N starvation by the plant and the alleviation of starvation by N_2-derived NH_4^+. Comparing physiological with transcriptional events led to a new understanding of the association, summarized in Figure 7. In brief, the expression of many genes is affected by N starvation only for the effect to be reversed at the onset of N_2 fixation when *N. punctiforme* is present. Expression of genes related to photosynthesis are transiently depressed, and expression of genes related to N acquisition are transiently increased. Similar to the latter, the increased expression of cytochrome P450 may serve to consume the excess reductant resulting from N starvation, but expression of the enzyme drops precipitously when N_2 fixation emerges to consume reductant. Finally, there is a substantial class of genes that respond to the presence of *N. punctiforme* well before the onset of N fixation. For example, the SWEET sugar transporter increases its expression immediately, dependent on the presence of the cyanobacterium. There has long been no doubt that *Anthoceros* spp. can influence the behavior of *Nostoc* spp. Now it is also evident that the signaling is bidirectional, independent of the ability of *Nostoc* spp. to fix N_2.

Fig. 7.
Summary of the known and inferred temporal metabolic responses and interactions between *Anthoceros punctatus* and *Nostoc punctiforme* during nitrogen starvation–induced establishment of a symbiotic association. The physiological landmarks across the top and bottom are formation and behavior of *N*. *punctiforme* hormogonia, transition from linear filaments in hormogonia primordia with around 8% heterocysts to aseriate filaments in symbiosis with about 30% heterocysts and fourfold higher nitrogenase activity (yellow highlight) and transient chlorophyll content of symbiotic *A*. *punctatus*. Across the bottom is the decline in chlorophyll of symbiont-free *A*. *punctatus*. Within the two respective boxes are the general patterns of gene expression. In cocultured (symbiotic) *A*. *punctatus*, expression of nuclear-encoded photosynthetic genes was initially downregulated and then upregulated in a pattern corresponding to the provision of dinitrogen-derived ammonium, but their transcription remained depressed in symbiont-free gametophytes. Chloroplast-localized glutamine synthetase showed a pattern similar to photosynthetic genes in both tissues, except its expression began to decline when nitrogenase activity also began to decline. Conversely, transcription of ammonium and nitrate transporters was initially upregulated in both cocultured and symbiont-free gametophytes and remained elevated in symbiont-free tissue but declined as fixed N became available to cocultured tissue. This pattern was repeated with a cytochrome P450 as representative of a reductant sink; its expression was transiently upregulated and then downregulated in the same pattern as cocultured tissue but remained elevated in symbiont-free gametophytes. The sugar transporter SWEET was upregulated only in cocultured tissue, independent of fixed N provided by *N*. *punctiforme*.

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These observations are noteworthy only if *N. punctiforme* can stand in reliably for natural symbionts, even though this cyanobacterium is unable to maintain stable, productive associations with *A. punctatus*. We believe this is the case for three reasons. First, the rate of acetylene reduction after initiation of coculture of *A*. *punctatus* with *N*. *punctiforme* is comparable to the steady-state rate from a natural symbiont *Nostoc* sp. strain PCC 9305 (Steinberg and Meeks 1991). Second, chlorophyll content recovers to normal levels. Third, the same is true for the recovery of PS II quantum yield. Thus, the physiological and, especially, the temporal RNA-seq data from days 0 to 14 can be interpreted in terms of the establishment of a functional N_2-fixing association with *Nostoc* spp.

The experimental system described here opens new possibilities for the investigation of plant-cyanobacterial symbioses, relying on the much-improved genome assembly presented here, a global view of *A. punctatus* gene expression, to complement already available information about *N. punctiforme* gene expression (Campbell et al. 2007, 2008; Christman et al. 2011). With the recent development of a hornwort transformation system (Frangedakis et al. 2021) adding to the ability to modify the genome of *N. punctiforme*, it is becoming feasible to carry out functional studies with plant genes.

MATERIALS AND METHODS

Plant and cyanobacterium growth conditions.

Sterilized spores were germinated to obtain axenic gametophyte tissues of *A. punctatus* (Enderlin and Meeks 1983). Hutner's minimal medium with NH_4NO_3 as the N source (H + N) was used for the growth of *A. punctatus* (Enderlin and Meeks 1983). The minimal medium was supplemented with 5 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) (Sigma Chemical Co.) adjusted to pH 6.4 with NaOH as buffer and 0.5% (wt/vol) glucose, originally included to increase the growth rate of light-limited laboratory cultures. Gametophyte tissues were incubated in 100 ml of medium in 300-ml Erlenmeyer flasks, at 20°C and 50 rpm orbital shaking, under 20-watt fluorescent lamps (3.5 to 8.0 W m^{-2}; cool-white) with a 16-h of light and 8-h of dark cycle. The stock cultures of symbiont-free *A. punctatus* were subcultured into H + N plus MES and glucose every 14 days.

N. punctiforme strain American Type Culture Collection (ATCC) 29133 (syn: Pasteur Culture Collection [PCC] 73102) was maintained under standard growth conditions (Enderlin and Meeks 1983). Due to random spontaneous mutation and absence of stringent selection, *N. punctiforme* and its ilk are prone to lose hormogonium gliding motility and, ultimately, hormogonium differentiation during serial transfer in liquid suspension laboratory culture; therefore, wild-type cultures are discarded after one year and a fresh culture is retrieved from −80°C storage. For experiments, the cultures were grown to an early light-limited linear phase in the standard minimal salts medium, diluted fourfold, with N_2 as the N source (Enderlin and Meeks 1983). The cultures were incubated in 50 ml of medium in a 125-ml Erlenmeyer flask with orbital shaking at 120 rpm and 25°C under 19 to 46 W m^{-2} s^{-1} of cool-white fluorescent light. Chl *a* content of *N. punctiforme* was determined after extraction in 90% methanol (Meeks and Castenholz 1971).

A. punctatus–N. punctiforme symbiotic reconstitution and sampling.

Gametophyte tissues of *A. punctatus*, grown in H + N were washed with Hutner's medium lacking combined N (H-N) and were transferred to two 100 ml of H-N medium, with MES and glucose (in 300-ml Erlenmeyer flasks). Approximately 10 to 12 g FW of *A. punctatus* gametophytes (14 days after the last transfer) were added to each of the two flasks. One was cocultured with 100 to 135 μg Chl *a* content of *N. punctiforme*, whereas the other flask was symbiont-free. The flasks were incubated under the growth conditions described above. The gametophytes were sampled at day 0 (before initiation of the N starvation) and after 2, 5, 7, 10, 14, and 28 days of incubation. The whole experiment was replicated three times, with a total of 39 samples.

Acetylene reduction assay, total Chl estimation, and Chl fluorescence measurements.

Nitrogenase activity was monitored by the reduction of acetylene to ethylene. Approximately 100 mg of tissue was sampled and washed, for approximately 2 min, using streams of distilled water to remove epiphytic attachment of *N. punctiforme* (as were all cocultured tissues prior to experimental analysis). The cleaned sample was incubated in 2 ml of H-N medium in a 7.5-ml glass vial sealed with a septum stopper. Acetylene was made freshly from calcium carbide (CaC_2) and was injected into each vial to 6% (vol/vol). The ethylene-acetylene content was monitored by gas chromatography after 30, 60, and 90 min of incubation. One hundred microliters of a sample from the vial atmosphere were injected onto a Porapak R column in a gas chromatograph equipped with a flame ionization detector (model 940; Varian Instrument Division, Walnut Creek, CA, U.S.A.). The normalization and calculation of the rate of ethylene production using the excess acetylene as an internal standard (i.e., ratio of ethylene to acetylene equals the amount of ethylene produced based on a standard curve) were performed according to Steinberg and Meeks (1991). The number of *N. punctiforme* colonies in cocultured gametophytes was determined under a stereomicroscope.

The total Chl *a* and Chl *b* content from N-starved cocultured and symbiont-free gametophytes was estimated after extraction of 100 mg (freshly ground sample in liquid nitrogen [LN]) of plant sample in 10 ml of 80% acetone and was quantified using the dichromatic equations of Inskeep and Bloom (1985).

Dark-adapted (20 min darkening) Chl fluorescence yield (F_v/F_m) was estimated with an imaging PAM fluorometer (PAM-2000; Walz, Effeltrich, Germany) with a saturating pulse (7,000 μmol m^{-2} s^{-1} for 1 s) of blue light to measure the maximum dark-adapted fluorescence yield F_m. The maximum and effective quantum yields of PS II electron transport were calculated as $F_v/F_m = (F_m - F_0)/F_m$.

All the above-mentioned experiments were repeated three times (biological replicates), with three technical replicates for each timepoint.

Improved *A. punctatus* genome assembly and annotation.

Following updates to Oxford Nanopore's Guppy basecaller, the raw sequence data used to assemble the first iteration of the *A. punctatus* genome (Li et al. 2020) was re-basecalled and assembled with Flye (Kolmogorov et al. 2019). The draft assembly was polished with five iterations of Pilon (Walker et al. 2014), using 100× coverage. A custom repeat library was created with EDTA (Ou et al. 2019) and input into RepeatMasker (Smit et al. 2015), to mask repetitive regions of the genome for gene annotation. Gene models were predicted with BRAKER2 (Brůna et al. 2021), using as training data RNA-seq data from one replicate of the Li et al. (2020) cyanobacterial symbiosis experiment as well as the proteins they predicted from *A. agrestis*. Functional annotations of genes were obtained using eggNOG-mapper (Cantalapiedra et al. 2021). The complete set of called genes and their annotations can be found in Supplementary Datasets S1 and S2, respectively. Genome annotation completeness was measured using BUSCO v5 with the Viridiplantae odb10 reference dataset (Simão et al. 2015).

Plant RNA extraction and RNA sequencing protocol.

The freshly sampled and washed gametophytes were ground in LN with a mortar and pestle and were stored at −80°C. RNA was extracted from 100 mg of frozen tissue using the Spectrum total RNA plant kit (Sigma-Aldrich). The mRNA library was prepared by poly-A enrichment and paired-end sequencing was performed on a Illumina NovaSeq6000 (2 × 150 bp) by Novogene (Sacramento, CA, U.S.A.).

RNA-seq data set and sequencing analysis of *A. punctatus–N. punctiforme* symbiosis.

All FASTQ files were quality-filtered and trimmed using fastp version 0.20.0 (Chen et al. 2018b) and were subsequently mapped to the reference genome using HISAT2 version 2.1.0 (Kim et al. 2015). All reads were 96.33 to 98.57% mapped to the genome. Transcript abundances were estimated using the updated *A. punctatus* genome annotation in Stringtie version 2.1.1 (Pertea et al. 2015).

The median of ratios method of normalization was used to normalize all the gene counts (Anders and Huber 2010), which were later used to plot graphs of different temporal gene expression patterns from selected clusters and expression patterns from a few selected genes. The raw gene counts can be found in Supplementary Dataset S1 and the normalized counts for DEGs can be found in Supplementary Dataset S2. Variance stabilizing transformation–normalized genes were fed into maSigPro (Conesa et al. 2006) in R version 4.0.2, to analyze the changes in the temporal gene expression patterns. Those genes with significantly different expression patterns between the two culture conditions ($P \leq 0.05$) (DEGs) can be found in Supplementary Dataset S3. The DEGs were clustered into nine different groups according to their gene expression patterns, using hclust in maSigPro. The gene location numbers are formally assigned as, for example, Anthoceros_punctatus_v2_contig1_g00020; on occasion, we refer to the location numbers in figures, tables, or text as, for example, contig1_g00020 or g00020.

Organization of the functional annotations of DEGs into metabolic categories was based on an approach applied to analysis of *N. punctiforme* transcriptomes involved in cellular differentiation alternatives (Campbell et al. 2007). Identification of functional hierarchies was accomplished by similarity searches into specific (i.e, EcoCyc, TAIR) and general (i.e., Interpro, Uniprot, National Center for Biotechnology Information [NCBI]) internet genome analyses sites. A detailed description of the functional hierarchies can be found in Supplementary Note S1.

Statistical analysis.

The results of cocultured or symbiont-free *A. punctatus* gametophytes in acetylene reduction assays, total Chl content, and F_v/F_m were tested using analysis of variance, followed by Tukey's HSD test with the function aov and tukeyhsd, using the packages “ggpubr”, “agricolae” part of R core package stats, version 4.0.2 (R Core Team 2020). PCA was conducted in R studio version 4.0.2 (R Core Team 2020) with package ggplot in DESeq2 (Love et al. 2014). To perform PCA, the best-fitting line was determined in 23,021-dimensional space (normalized expression values for the 23,021 *A. punctatus* genes) through the 39 points representing each condition (three replicates, six timepoints, two culturing conditions plus one common time zero). That line minimized the sum of the distances from each point to the line and thereby maximized the variance captured. Then a second line was determined, perpendicular to the first, that captured as much of the remaining variance as possible. The two lines served as two-dimensional axes (principal components) that captured 57% of the total variance. There is a risk of missing insights by ignoring 43% of the variance, but this is outweighed by the simplification of compressing thousands of axes into just two.

Data availability.

Raw sequence reads for the RNA sequencing have been deposited at the NCBI Short Read Archive and GenBank with accession number PRJNA750572.

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