Measurement of a possible signal for nitrogen starvation in a cyanobacterium-plant symbiosis

I. Introduction

One of the greatest advances in agriculture (and thus in human civilization) occurred in the middle of the 20th century, when strains of crop plants were developed that allowed considerably greater yields, at the expense of high inputs of nitrogenous fertilizer.[1] The resulting increase in the use of nitrogenous fertilizers came at a great cost, however. As much as 1% of the total global energy budget is used to produce a single chemical - ammonia.[2,3] Just as important, the use of nitrogenous fertilizers has wreaked havoc on the environment, as the eutrophication of waterways fed by agricultural runoff has drastically altered their ecology.[4]

Certain crop plants, the legumes (e.g. beans and alfalfa), can meet all of their nitrogen needs through the fixation of atmospheric N_2, thus avoiding the economic costs of synthesizing ammonia and the ecological costs of applying it. The legumes are able to do this by forming symbioses with certain N_2-fixing bacteria, the rhizobia. However, legumes constitute only a small fraction of the world's agricultural output (**Fig. 1**).[5] The rest, particularly the cereals rice, wheat, and corn, rely on fertilizer to achieve maximum yields.

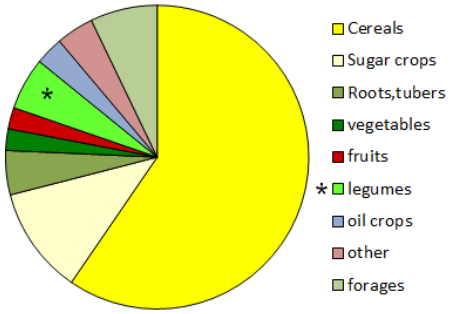

Figure 1: Fraction of world agriculture allocated to different crop classes (mid 1990's). Adapted from Table 1 of Ref 5.

Many have considered different strategies to extend the benefits of biological N_2-fixation to crop plants beyond the legumes.[6,7] One idea, creating novel symbioses between cereals and rhizobia is currently far out of reach, as the rhizobia are generally quite specific in the plants they infect.[8] The same is not true, however, of *Nostoc*, a N_2-fixing cyanobacterium. This bacterium enters into productive symbioses with the broadest range of plants: both monocots and dicots, as well as simpler plants and fungus.[9] This relative lack of discrimination probably arises from the fact that *Nostoc* is intrinsically able to fix N_2 by differentiating specialized N_2-fixing cells called heterocysts.[9] Rhizobia are quite the opposite, requiring considerable aid from the plant host to fix N_2. They do not do so under natural conditions outside the plant.[10] Although *Nostoc* is not known to form symbioses with plants of agricultural importance, it might be easier to find a way to create symbioses between cereals and *Nostoc* than between cereals and rhizobia.

While *Nostoc* fixes N_2 both in its free-living and symbiotic states, there are important differences. Studies following the fate of isotopically marked N_2 gas indicate that free-living *Nostoc* retains almost all of the nitrogen it fixes.[11] In contrast, a similar isotopic study showed that the cyanobacterium in association with plants releases up to 90%.[12] Furthermore, the level of ammonia produced by nitrogen fixation in the plant is vastly higher than the level sufficient to inhibit nitrogen fixation in free-living *Nostoc*.[13] Evidently the plant environment alters the way that *Nostoc* perceives fixed nitrogen, but the mechanism by which it does so is far from clear.

A key to resolving this mystery may reside in the metabolite α-ketoglutarate (also called 2-oxoglutarate), which plays a central role in N_2-fixation, as shown in **Fig. 2**.[14] This compound is the ultimate source of carbon that is added to the fixed nitrogen and is the last non-nitrogenous compound in the pathway feeding carbon to the glutamine/glutamate cycle (**Fig. 2**). If that cycle

is not operating, the cell may starve for nitrogen, and at the same time α-ketoglutarate should accumulate.

Li et al (2003) speculated that α-ketoglutarate might be a molecular signal that indicates to the cell that it is starving for nitrogen.[15] They tested this idea by constructing a strain of *Nostoc* that expressed a gene from *E. coli* that encodes a protein capable of transporting α-ketoglutarate through the cell membrane. This strain responded to a growth medium containing α-ketoglutarate outside the cell by differentiating N_2-fixing heterocysts even in the presence of a level of fixed nitrogen that would otherwise prevent heterocyst differentiation. The presence of a high level of α-ketoglutarate within the cell evidently tricked *Nostoc* into making heterocysts when they were not necessary for growth, consistent with a role for α-ketoglutarate as a signal of nitrogen starvation.

Conceivably, plants with whom *Nostoc* enter into symbioses do the same thing, using α-ketoglutarate to fool *Nostoc* into continuing to fix N_2 even when it does not need the fixed nitrogen for its own growth. The purpose of the experiment described in this proposal is to test whether this is the case.

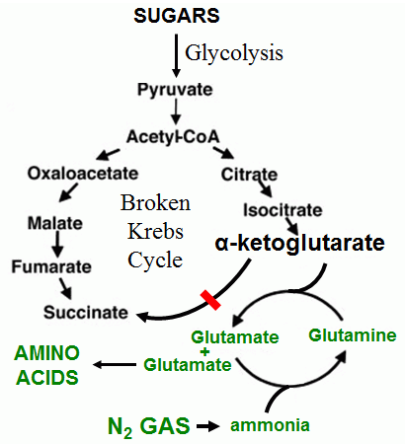

Figure 2: Metabolism of nitrogen fixation in *Nostoc*. Carbon metabolism is shown in black, and metabolism related to N-fixation is shown in green. Cyanobacteria lack a complete citric acid cycle, and so α-ketoglutarate may accumulate in response to the activity of nitrogen-fixation (adapted from Ref 14).

II. Experiment

The aim of this experiment is to determine the concentration of α-ketoglutarate in *Nostoc* when the cyanobacterium is growing within a plant host and to compare that concentration with the level of α-ketoglutarate within free-living *Nostoc* grown with different levels of fixed nitrogen. If the plant host is somehow manipulating the level of α-ketoglutarate, then I would expect that the level of α-ketoglutarate within the symbiosis would be similar to that found in free-living *Nostoc* grown without any nitrogen source besides N_2, i.e. under conditions that *Nostoc* would interpret as N-starvation.

II.A. Biosensors

The amount of *Nostoc* within the plant is too small to permit routine measurement of metabolites, so the level of compounds in this experiment will be determined by the highly sensitive fluorescent resonance energy transfer (FRET) method[16] illustrated in **Fig. 3**.[17] Some biosensors work as shown in the figure: binding of the metabolite decreases the distance between the fluorophores and increases the fluorescence of the second fluorescent

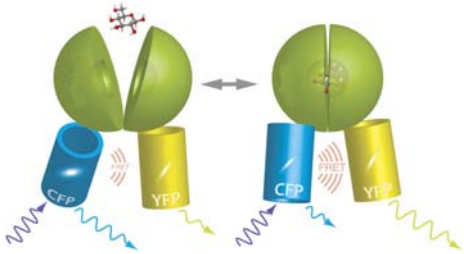

Figure 3. Principle behind FRET biosensors. The method relies on an artificial protein composed of a two-domain binding protein (green) and two fluorescent proteins, in this example cyano fluorescent protein (CFP, blue) and yellow fluorescent protein (YFP, yellow). When the target metabolite is absent, CFP and YFP are distant from each other. As a result, irradiation using a wavelength absorbed by CFP results in blue fluorescence. When the specific binding protein binds the target metabolite, the proteins conformation changes, bringing CFP and YFP close to each other. As a result, the blue fluorescence emitted by CFP is absorbed by YFP, which thereupon emits yellow fluorescence. Thus, the amount of yellow fluorescence is diagnostic of the amount of target metabolite bound. Adapted from Ref. 17)

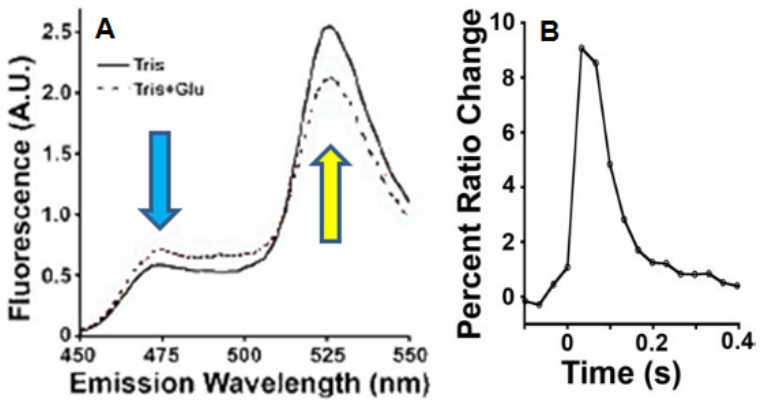

Figure 4. Fluorescence of a recombinant glutamate (glu) - binding protein. (**A**) In vitro fluorescent emission was measured in Tris buffer with or without glutamate. The blue and yellow arrows indicate the peak emission of CFP and YFP, respectively. (**B**) The ratio of peak CFP emission to peak YFP emission was determined in cultured hippocampal neurons expressing the protein. At time 0, an electric pulse induced the release of glutamate neurotransmitter. (Adapted from Ref 18).

protein. Other biosensors go the other way: binding causes an *increase* in distance and a *decrease* in fluorescence by the recipient fluorophore.

This method was employed by Hires et al (2008)[18] to detect glutamate at the surface of hippocampal neurons stimulated to release neurotransmitter. They first constructed a gene derived from that encoding a glutamate/aspartate-binding protein from *E. coli*, in which the gene was fused at the N-terminus with the gene encoding cyano fluorescent protein (CFP) and at the C-terminus with the gene encoding Citrine, a yellow fluorescent protein (YFP). The recombinant protein fluoresced at two peak wavelengths in response to light absorbed by CFP: a blue emission peak at about 475 nm and a yellow emission peak at about 525 nm (**Fig. 4A**). The presence of glutamate increased blue emission at the expense of yellow emission, presumably because the two fluorescent proteins moved away from each other when the intervening glutamate-binding region of the protein bound glutamate. The ratio of emission at 475 to emission at 525 was used as a measure of glutamate levels outside of cultured hippocampal neurons into which the recombinant gene had been introduced. Inducing the release of neurotransmitter led to a rapid but transient rise in the fluorescence ratio, hence in the external level of glutamate (**Fig. 4B**).

Many other similar biosensors have been constructed,[16] but unfortunately none have been reported (to my knowledge) that are sensitive to α-ketoglutarate. I'll therefore use biosensors for molecules metabolically related to α-ketoglutarate: glutamate and glutamine. Different biosensors respond to different levels of target metabolite. Hires et al (2008) reported mutants of their construct that were sensitive to different levels of glutamate, ranging from 2.5 μM to 700 μM.[18] It is difficult to guess which would be the most appropriate to use in *Nostoc* cells. Yang et al (2010) described a collection of glutamine biosensors with a wide range of binding affinities.[19]

II.B. Introduction of biosensors into plant/*Nostoc* symbiosis

Genes encoding the glutamate and glutamine biosensors will be cloned into a plasmid, pRL502, that facilitates the expression of foreign genes in *Nostoc*.[20] The plasmid will be introduced to *Nostoc punctiforme* by means of conjugation as previously described.[21] The resulting strain, expressing one of the two biosensors, will be grown with the plant *Anthoceros* (**Fig. 5**) to bring about a symbiotic association.[22]

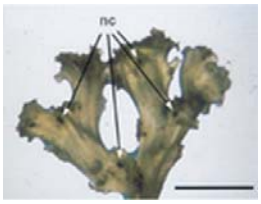

Figure 5. *Anthoceros punctatus.* *Nostoc* colonies (nc) shown. Bar is 1 cm. (from Ref. 9)

Levels of glutamate and glutamine will be estimated by measuring the ratio of the peak CFP and YCP fluorescence (using a hypothetical fluorometer) and comparing that ratio to a standard curve obtained by incubating the isolated protein with glutamate and glutamine in vitro. Also, the ratio will be measured in free-living *Nostoc* grown in different levels of ammonia under an argon/CO_2 atmosphere (to avoid N_2-fixation). In this way I can control to some extent the degree of internal nitrogen starvation.

III. Discussion

If all goes well, the fluorescence ratios using both the glutamate and glutamine biosensors will exhibit clear differences between *Nostoc* grown with and without fixed nitrogen, and the ratios in *Nostoc* grown symbiotically with the plant will lie within that range. From this information I might be tempted to draw some conclusion regarding the concentrations of these two amino acids, perhaps rashly (see below). Unfortunately, Even if this were possible, it is not clear how the experiment will test whether α-ketoglutarate serves as an internal signal for nitrogen starvation. The model (**Fig. 2**) makes no clear predictions concerning the concentrations of glutamate and glutamine.

The problem is that what I ***really*** want is an α-ketoglutarate biosensor. Though it may not exist, the materials may be at hand to construct it. At least three proteins bind α-ketoglutarate to a degree that may serve. The citric acid cycle enzyme α-ketoglutarate dehydrogenase is possible, though binding of the substrate is complicated.[23] The bacterial sensor protein PII also binds α-ketoglutarate, and its crystal structure is known,[24] which would be very helpful in guiding the construction of a biosensor.[25] The same is true with another bacterial sensor protein, NtcA.[26] Unfortunately, both PII and NtcA proteins are important metabolic regulators in *Nostoc* and introducing biosensor derivatives based on either one of them may well perturb the system. In any case, making a new biosensor would be a very time consuming project, well outside the scope of this proposal. The proposed experiment must be viewed as laying the ground work for the more informative experiments that may follow.

It will be difficult enough to interpret the results with the glutamate and glutamine biosensors. *Nostoc*, like all cyanobacteria, is highly fluorescent by itself,[27] and this may interfere with the detection of fluorescence from the biosensors. Secondly, the biosensors may not be sensitive to the levels of glutamate and glutamine in the cell. This is a soluble problem, though one that is tedious to address, since there are many derivatives of the biosensors sensitive to a wide range of concentrations.[18,19] It is also possible that the glutamate biosensor will be confused by cellular levels of aspartate, since the protein was based on a glutamate/aspartate binding protein.[18] More problematic is the observation that the fluorescence ratio varies in response to the ionic strength of the medium.[18] The apparent concentration of the metabolite indicated by the measured fluorescence ratio may therefore be misleading, owing to a difference between the ionic strength in the cell and that of the in vitro medium used in the construction of the standard curve.

Despite these problems, biosensors offer a powerful tool to assess metabolite concentrations within the cell. With appropriate care, they may shed light on signaling mechanisms underlying the development of productive symbioses between plants and N_2-fixing cyanobacteria. If the mechanism turns out to be relatively simple, it might be possible to modify important crop plants to host N_2-fixing cyanobacteria, thereby reducing our current reliance on expensive and ecologically damaging nitrogenous fertilizer.

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