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Association of N_2-fixing cyanobacteria and plants: Towards novel symbioses of agricultural importance

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Scientific Findings

Introduction

A sizable fraction of the world's available energy is expended in the chemical production of combined nitrogen from N_2 and its application as fertilizer to maximize agricultural output. Some crops, legumes such as beans and alfalfa, provide maximal yields without applied nitrogen, because of nitrogen-fixing bacteria residing in their roots. It has long been a goal to extend the benefits of biological nitrogen fixation to other crop plants, particularly the grains.

The current project stemmed from the observation by one of the senior researchers that a strain of nitrogen-fixing cyanobacteria, *Nostoc* 2S9B, is able to infect the roots of wheat and spread to other parts of the plant [Gantar et al., 1991], and that nitrogen fixed by the strain can support the growth of the plant [Gantar et al., 1995]. Since predation and competition limit the external concentration of cyanobacteria in the field, we felt that it was important to determine the extent to which internalized *Nostoc* was able to meet the nitrogen needs of wheat and to investigate means by which the levels of infection could be raised. Finally, we wished to develop molecular tools that would enable us to follow the course of infection and also to investigate the physiological parameters that enable this strain but not others to form tight associations with wheat.

DISCLAIMER

K. DESCRIPTION/ABSTRACT

Some nitrogen-fixing cyanobacteria are able to form symbioses with a wide variety of plants. *Nostoc* 2S9B is unusual in its ability to infect the roots of wheat, raising the prospect of a productive association with an important crop plant. The goal of the project was to lay the groundwork for the use of novel associations between *Nostoc* and crops of agronomic importance, thereby reducing our reliance on nitrogenous fertilizer. *Nostoc* 2S9B was found to enter roots through mechanical damage of roots and reside primarily in intercellular spaces. The strain could also be incorporated into wheat calli grown in tissue culture. In both cases, the rate of nitrogen fixation by the cyanobacterium was higher than that of the same strain grown with no plant present. Artificial nodules induced by the action of hormone 2,4D were readily infected by *Nostoc* 2S9B, and the cyanobacteria within such nodules fixed nitrogen under fully aerobic conditions. The nitrogen fixed was shown to be incorporated into the growing wheat seedlings. *Nostoc* thus differs from other bacteria in its ability to fix nitrogen in *para*-nodules without need for artificially microaerobic conditions.

It would be useful to introduce foreign DNA into *Nostoc* 2S9B in order to make defined mutations to understand the genetic basis of its ability to infect wheat and to create strains that might facilitate the study of the infection process. Transfer of DNA into the cyanobacterium appears to be limited by the presence of four restriction enzymes, with recognition sequences the same as *Bam*HI, *Bgl*I, *Bsa*HI, and *Tth*111I. Genes encoding methyltransferases that protect DNA against these four enzymes have been cloned into helper plasmids to allow transfer of DNA from *E. coli* to *Nostoc* 2S9B.

Mode of infection of wheat roots by *Nostoc* 2S9B

A major objective of the project of the project was to optimize the infection of wheat roots by *Nostoc,* and it seemed prudent to characterize the means by which infection naturally occurs. The basal level of infection was determined to be a rather low 1 or 2 cyanobacterial packages per 100 cells on the surface of the plant. The strain that had been used in earlier work was not axenic, and the possibility that the infection by *Nostoc* required the aid of other bacteria, however, axenic *Nostoc* infected with equal capability, so the property resides with the cyanobacterial strain alone.

Since we had already seen that *Nostoc* appears to enter the plant through spaces between plant cells at the surface of roots (Ow et al., 1999), we considered that mechanical damage of roots might be a means of increasing cyanobacterial entry. A short period of sonication of wheat roots increased the number of internal cyanobacterial packages by a factor of nearly 20-fold and had only a small effect on root growth. However, internal cyanobacterial biomass was only slightly increased by the procedure. Other factors besides entry, such as availability of carbon, might limit the level of cyanobacteria within the plant.

A report of this work has been published (Gantar, 2000b).

Incorporation of *Nostoc* into wheat grown in tissue culture

To avoid the limitations imposed by the entry process into mature plants, we turned to wheat grown in tissue culture. Wheat calli cocultivated with *Nostoc* 2S9B readily took up the cyanobacterium. The cyanobacterium was found both between cells within calli and within cells. As in natural symbioses between *Nostoc* strains and plants, the cyanobacterium tended to reside in intercellular regions filled with polysaccharide. The *Nostoc* within the callus had a heightened specific activity of nitrogenase and level of growth relative to cultures grown in the absence of calli. However the regeneration of mature wheat plants from calli was inhibited by the presence of cyanobacteria. Before cocultivation in tissue culture can be used as a means to introduce *Nostoc* into wheat, it is necessary to determine the mechanism by which the cyanobacterium interferes with plant differentiation.

A report of this work has been published (Gantar, 2000a).

Colonization of artificial nodules by *Nostoc*

Many workers have sought to extend the benefits of biological nitrogen fixation by the Rhizobia to plants outside of their natural hosts, the legumes, by induction of *para*-nodules, artifical cavities formed on plants through the action of the hormone 2,4-D (Kennedy et al., 1997). Unfortunately, while Rhizobia can infect *para*-nodules and express nitrogenase under artificially microaerobic conditions, the amount of nitrogen fixed under normal aerobic conditions is very low. This is to be expected, since Rhizobia

require a specialized environment provided by the plant to maintain the low oxygen levels required by nitrogenase.

Since *Nostoc* is able to fix N_2 even under fully aerobic conditions, owing to its ability to produce specialized nitrogen-fixing cells, heterocysts, that maintain internal microaerobiosis, we reasoned that the cyanobacterium would be a more likely candidate to form productive infections of *para*-nodules. Wheat seedlings induced to form *para*-nodules by 2,4-D took up about 4-times more *Nostoc* 2S9B than untreated seedlings. Nitrogenase activity tightly associated with the plant was similarly increased by the hormone, indicating that the *para*-nodules formed in response to 2,4-D housed nitrogen-fixing cyanobacteria. This conclusion was consistent with ultrastructural observations.

The presence of both *Nostoc* and hormone led to a 3-fold increase in the percentage of root-associated nitrogen, after accounting for cyanobacterial biomass. The percentage of nitrogen in stems was not similarly affected, however. Biomass rather than percent nitrogen would be closer to the bottom line, but 2,4-D itself diminishes biomass. For *para*-nodules to be an effective means of housing nitrogen-fixing bacteria, plants would need to be genetically engineered to produce the structures in the absence of hormone.

A report of this work has been published (Gantar and Elhai, 1999).

Quantitation of transfer of nitrogen from internal *Nostoc* to wheat

While we (Gantar et al, 1995) and others (Spiller and Gunasekaran, 1991) have demonstrated that nitrogen-fixing cyanobacteria can support the nitrogen needs of a plant in the laboratory, the luxuriant growth of cyanobacteria grown under such conditions would not occur in the field. We wished to determine the degree to which *Nostoc* 2S9B internal to wheat could meet the needs of the plant. To that end, infected plants washed free of external *Nostoc* was exposed to $[^{15}N]N_2$ to permit the quantitation of incorporation of the produces of nitrogen fixation into plant tissue. These experiments are nearly completed.

Genetic manipulation of *Nostoc* 2S9B

We wished to introduce foreign genes into *Nostoc* 2S9B for two reasons: (1) to tag the strain and thus facilitate studies of the infection process, and (2) to mutate the strain in order to determine the molecular basis for its unique ability to infect wheat. We learned early on that conventional approaches to introduce DNA into the strain were not up to the task. We found that the strain possesses four restriction enzymes, more than enough (Elhai et al, 1997) to explain the absence of DNA transfer we observed.

In response to the difficulties posed by host restriction, we adopted two parallel strategies. First, we developed a novel means of overcoming restriction, based on the ability of T4 phage of *E. coli* to hydroxymethylate C residues of plasmids carrying the phage origin of replication (Kreuzer et al., 1988). We created a plasmid vector that can

replicate in a wide range of bacteria and also contained a T4 origin of replication enabling it to be replicated and hydroxymethylated by phage T4. The plasmid was successfully electroporated into *Nostoc* 2S9B but could not be propagated stably. This work was reported at an international meeting (Matveyev et al., 1997).

The second approach followed that used by those working with another cyanobacterium, *Anabaena* PCC 7120 (Elhai, et al, 1997). After characterizing the specificities of the four restriction activities, we created two helper plasmids that between them carried DNA methyltransferases that protected against all four restriction activities. Plasmids designed to replicate in both *E. coli* and *Nostoc* become resistant to restriction by the *Nostoc* 2S9B enzymes when they are passed through a strain of *E. coli* carrying the helper plasmids. We are now in the process of testing the degree to which the helper plasmids can increase the rate of conjugation of plasmids from *E. coli* to *Nostoc* as well as using them to introduce transposons for mutagenesis.

Publications based on work supported through the grant

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