Tough love: accommodating intracellular bacteria through directed secretion of antimicrobial peptides during the nitrogen-fixing symbiosis

Christina Stonoha-Arther[1] and Dong Wang[1,2]

The symbiosis formed by nitrogen-fixing bacteria with plant hosts mainly in the legume family involves a very intimate interaction. Within the symbiotic organ (the nodule) the bacteria are fully internalized by the host cell to become an intracellular organelle surrounded by a host-derived membrane. This arrangement is probably necessary for the efficient provision of energy and the sequestration of free oxygen molecules, two conditions required for sustained nitrogen fixation. Recent advances made in model legume species, such as *Medicago truncatula*, are beginning to uncover the genetic components allowing rhizobia to access the host cytoplasm and establish chronic intracellular infections without overt detrimental effects. It is now known that the rhizobial compartment in *M. truncatula* cells, the symbiosome, retains some features of the extracellular space as the target for a redirected host protein secretory pathway. A set of vesicle trafficking proteins function specifically in symbiotic cells to ensure the faithful delivery of secretory proteins to the intracellular bacteria, or bacteroid. This system is co-opted from the more ancient association with arbuscular mycorrhizal fungi found in most land plants, highlighting the evolutionary origin of the legume–rhizobia symbiosis. In some legume lineages, this heightened capability to process secretory proteins is needed to deliver a large number of symbiosis-specific antimicrobial peptides to the bacteria. Known as NCR peptides, these molecules transform bacteroids into a state of terminal differentiation, where the microbe loses its ability to proliferate outside their host. Numbering in their hundreds, these peptides manipulate various aspects of rhizobial biology, and affect the outcome of this symbiosis in complex ways. The extreme size of the NCR peptide family seems to be the result of an evolutionary conflict between the two partners to extract maximum benefit from each other.

Addresses
[1] Plant Biology Graduate Program, University of Massachusetts Amherst, MA 01003, USA
[2] Department of Biochemistry and Molecular Biology, University of Massachusetts Amherst, MA 01003, USA

Corresponding author: Wang, Dong (dongw@biochem.umass.edu)

Introduction

Some legume plants form a mutualistic relationship with soil bacteria to utilize atmospheric nitrogen which is otherwise inaccessible to plants. The main benefit of nitrogen-fixing symbiosis for both organisms is an acquisition of nutrients. The plant host receives fixed nitrogen from the bacterial symbiont in the form of ammonia and the bacteria receive photosynthetic carbon [1].

Although the exact molecular dialogue between the plant and symbiont differs between species, the bacteria are ultimately internalized by the host and housed inside nodule cells. In all nodules, the internalized bacteria differentiate into bacteroids before they are able to fix nitrogen [2,3]. Some legumes, such as those in the inverted repeat-lacking clade (IRLC), including the model plant *Medicago truncatula*, cause the bacteroids to differentiate terminally [3,4].

In IRLC legumes, bacteroids are coaxed into more profound differentiation by the plant. This terminal differentiation consists of cell elongation, membrane permeabilization, cell cycle changes, and endoreduplication of the bacteria. This differentiation is irreversible, as it drastically reduces the ability of the bacteria to reproduce (hence the term 'terminal') [3]. Bacteroids undergo this transformation surrounded by a plant-derived symbiotic membrane. This organelle, containing the bacteroid and

the enveloping membrane (including the space between them) are collectively named the symbiosome. Symbiosome formation is the result of bacterial release from the infection thread, a long and narrow tubular invagination of the plasma membrane. In some less evolved systems of the symbiosis, such as in basal legumes and the non-legume *Parasponia*, rhizobia are not released, but remain in a modified infection thread called the fixation thread to fix nitrogen [5].

Bacteroid differentiation is dependent on the ability of the plant to traffic proteins to the membrane-bound bacteria inside the nodule cells [6]. This can be tricky because the newly formed membrane that surrounds the bacteria is derived from the plasma membrane but requires a distinct identity. The work of differentiation is performed by nodule-specific cysteine-rich (NCR) peptides [7], of which *M. truncatula* has a predicted 700, that are delivered to the symbiosome membrane. The exact role of every NCR peptide is unknown, but they seem to have antimicrobial effects *in vitro*, while being necessary for successful symbiosis *in planta*.

The ability of these NCR peptides to differentiate the symbionts is dependent on their accurate and successful delivery to the symbiosome by the plant's protein trafficking machinery [7]. Without the protein trafficking machinery or NCR peptides, the bacteria will not differentiate and will therefore not fix nitrogen for the plant [6]. This review will focus on protein trafficking of NCRs within the nodules and emerging roles for NCR peptides beyond bacteroid differentiation.

Protein trafficking during the nitrogen-fixing symbiosis

Protein trafficking genes involved in multiple steps of the anterograde protein trafficking pathway have been implicated in the symbiosis. A component of the signal peptidase complex (SPC), DNF1, was identified in *M. truncatula* through a forward genetics screen. Without *DNF1*, the nodules are small and nonfunctional because the bacteria in symbiosomes of this mutant do not differentiate into nitrogen-fixing forms [6]. The SPC is responsible for cleaving the signal peptide sequence from secretory proteins (including NCR peptides) entering the endoplasmic reticulum (ER). *DNF1* encodes SPC22, an essential subunit of the complex. In the *dnf1* mutant, these proteins retain their signal peptides and are trapped in the ER instead of being trafficked through the ER to reach the symbiosome. This suggests that proper secretion of host proteins to the symbiosome is crucial for the functioning of the nitrogen-fixing symbiosis. Several other protein-trafficking genes, including ones encoding the other SPC subunits, are similarly upregulated in the nodule [6,8], further emphasizing the importance of host protein secretion to the intracellular bacteria.

Protein secretion is a universal cellular function, and SPC22 in particular is essential for viability in eukaryotes. However, the *dnf1* mutant shows no other defects, that is, its effect is specific to symbiosome maturation. How could this be? A closer look at the *M. truncatula* genome provided the likely answer, in a close homolog sharing over 80% protein sequence identity with DNF1 [6]. This protein, DNF1L, is apparently sufficient to handle housekeeping levels of secretion to the extracellular space, allowing DNF1 to specialize in symbiosis. It is possible that DNF1 and DNF1L have evolved to function in different processes. However, given how similar they are at the protein level, it is more likely that DNF1 and DNF1L are functionally analogous, and that the true role of *DNF1* is to simply serve as a second gene copy of SPC22, to increase the secretory capacity of cells hosting symbioses. In other words, the specificity of the *DNF1* gene may lie in its transcriptional regulation rather than its protein sequence. Plant SPCs are made of four subunits (SPC12, SPC18, SPC22, and SPC25), each usually encoded by small families [9]. Similar to SPC22, one member each of SPC12 and SPC25 are also upregulated in *M. truncatula* nodules [6].

If this hypothesis is correct, then in symbiosome-containing cells, one single (albeit enhanced) secretory system handles cargoes destined for two destinations, the regular extracellular space and the new symbiosome. These are two functionally dissimilar compartments. For example, compared with the extracellular space, the symbiosome lacks a plant cell wall; its membrane contains a plethora of channels and pumps for molecular exchange between the partners in both directions; and it is home to a vast amount of proteins interacting with the bacteria [10]. It is possible that symbiotic cells redirect their flow of secretory proteins, making the symbiosome membrane the default target, in a manner similar to cells hosting arbuscular mycorrhizal fungi [11]. At the same time, symbiosomes are long-lived organelles that degrade only when their host cells senesce. If proteins are actively trafficked to both membrane compartments, cargo proteins in these cells must be steered to the correct compartments in a highly reliable manner. How is the separate flow of molecules to the extracellular space and the symbiosome accomplished in the same cell?

At the cellular level, this issue of specificity must be addressed at multiple stages of protein secretion. On one hand, cargoes must be packaged into different vesicles. On the other hand, the two target membranes — one marking the cell boundary and the other surrounding intracellular bacteria — must somehow be distinguished. Solutions to the latter issue is partially accomplished with a t-SNARE protein, an isoform of SYP132, that distinguishes the symbiosome membrane from the plasma membrane [12••,13••]. The SYP13 sub-family of t-SNAREs are well established markers for the plasma