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DOI: 10.1016/j.cub.2005.03.006

Legume Haemoglobins: Symbiotic Nitrogen Fixation Needs Bloody Nodules

How do plants create an environment in which symbiotic bacteria can reduce enough N_2 to provide the plant with sufficient ammonium for growth? Gene silencing has now been used to show that legume haemoglobins are crucial.

J. Allan Downie

Only bacteria contain the nitrogenase enzyme that can reduce N_2 to ammonium and so, during nitrogen-limited growth, some plants enter into a symbiotic interaction with nitrogen-fixing bacteria, which provide the plants with ammonium. A major problem in maintaining a high rate of nitrogen fixation, however, is that the bacterial nitrogenase enzymes are very oxygen sensitive, but at the same time require high levels of ATP to drive the reaction. So ideally, the bacteria require a high flux of oxygen to enable high rates of ATP synthesis, whilst simultaneously maintaining a low free oxygen environment to prevent inactivation of nitrogenase by oxygen. These paradoxical requirements are met by the formation of root nodules in which legumes provide an appropriate niche for rhizobia, the bacteria that differentiate into forms, known as bacteroids, which fix nitrogen in nodules. A crucial part of this niche is the presence of plant haemoglobins in the cytoplasm of the plant cells containing the bacteroids.

If you dig up the roots of a legume such as pea or bean and cut into one of the nodules on the root you will see that it has a blood-red colour (Figure 1, top). This is due to the high levels of haemoglobins, referred to as leghaemoglobins because they are always found in legume nodules [1]. As they report in this issue of *Current Biology,* Ott *et al*. [2], using an RNA interference (RNAi) approach to silence the expression of the three nodule-expressed leghaemoglobin genes in the legume *Lotus japonicus*, have now demonstrated that leghaemoglobins really are essential for symbiotic nitrogen fixation in legume root nodules.

Whereas animal haemoglobins in blood facilitate oxygen transfer between cells and organs, leghaemoglobins function in a manner more analogous to animal myoglobin [1], which facilitates oxygen transfer within the cytoplasm to mitochondria. Leghaemoglobins, however, can have a twenty-fold higher affinity for oxygen than myoglobin [3]. The oxygen-binding characteristics of leghaemoglobins are unusual in that they have an extremely fast O_2 association rate and a relatively slow O_2 dissociation rate [1], and so can buffer the free oxygen concentration at around 7–11 nM.

Calculations based on the levels of oxygenation and concentration of leghaemoglobin in the nodule cytoplasm suggest that the concentration of leghaemoglobin-bound oxygen is around 70,000 times higher than the free oxygen concentration [1]. This provides a substantial buffering capacity that will be important for providing a high flux of oxygen for bacterial respiration. But the low levels of free-oxygen pose a challenge for bacterial respiration, and the nitrogen-fixing bacteroids deal with this by inducing a symbiosis-specific cytochrome oxidase with a very high affinity for oxygen [4].

Given these observations on the biochemistry and physiology of nitrogen fixation in nodules, it had been anticipated that silencing of leghaemoglobin expression would affect symbiotic nitrogen fixation, as observed by Ott *et al*. [2]. What was not anticipated, however, was the absence of bacterial nitrogenase in the bacteria within the nodules of the plants lacking leghaemoglobin. Although the measured levels of free oxygen in nodules of the plants lacking leghaemoglobin were somewhat higher than those seen in nitrogen-fixing nodules, there was still a low oxygen environment, particularly in the deeper layers of the nodules [2]. So it seems unlikely that the complete lack of nitrogenase expression can simply be explained by the lack of a low oxygen environment, which could be observed in at least some parts of the nodules.

How then can we explain the lack of induction of bacteroid nitrogenase in these nodules? Perhaps some form of ramped induction could be required. Possibly a rapid induction of the bacteroid nitrogenase and high-affinity oxidase might cause a problem if it resulted in a high rate of oxygen consumption that could not be sustained in the absence of the leghaemoglobin-oxygen buffer. Conversely, if the leghaemoglobin was induced before the specialised bacteroid oxidase then

the developing bacteroids could have difficulties in respiring.

Indeed, there is an ordered sequential pattern of induction of bacterial nitrogen-fixation genes in response to gradually decreasing levels of oxygen [5]. Furthermore, there is clear evidence of differential expression of leghaemoglobin genes in pea nodules. The nodules formed on peas have an indeterminate meristem. This means that, by sectioning along the longitudinal axis of a pea nodule, it is possible to observe different stages of development of the symbiosis from the initiation of nitrogen fixation near the growing meristem through the mature nitrogen-fixing zone to a senescent zone near the root (Figure 1).

Two distinct patterns of leghaemoglobin expression during nodule development in pea were observed by *in situ* hybridisation using leghaemoglobin gene-specific hybridisation probes [6]. It is of particular interest that the leghaemoglobin expressed in the infection zone and in the region of bacteroid differentiation (Figure 1, middle) had a lower affinity for oxygen [6] than the leghaemoglobins expressed in the mature nitrogen-fixing zone of the nodule (Figure 1, bottom). One possibility is that the sequential expression of these different leghaemoglobins may be important for the sequential induction of bacterial genes required for symbiotic nitrogen fixation.

Conversely, the induction of bacterial nitrogen fixation influences the pattern of leghaemoglobin gene expression in legumes. For example, the high oxygen-affinity leghaemoglobin normally expressed in the mature nitrogen-fixing zone in pea nodules is very much restricted in its pattern of expression if the nodules are induced by mutant bacteria unable to fix nitrogen [6]. This implies that there is co-ordinated development between the two symbiotic partners during induction of symbiotic nitrogen fixation.

An alternative explanation for the lack of nitrogenase induction in nodules lacking leghaemoglobin is that the leghaemoglobin may be playing some role other than simply providing an oxygen buffer. It has been clear for some time that some leghaemoglobin genes are induced early in the symbiotic interaction, well before there is any requirement for nitrogen fixation. Rhizobia produce a so-called Nod factor, which is required for infection of roots by rhizobia. Purified Nod factors can induce the expression of several genes required for early steps in root infection, even in the absence of bacteria [7]. Surprisingly, Nod factors can induce expression of a leghaemoglobin gene within one hour of addition [8]. Nod factor induction of a leghaemoglobin in *Lotus* roots requires at least one of the plant genes required for early responses to Nod factor signal transduction [9]. This implies a role for leghaemoglobin possibly during early stages of infection, several days before symbiotic nitrogen fixation occurs. At this stage we do not know if such leghaemoglobin expression is related to oxygen transfer or possibly some novel aspect of signalling.

It has recently been reported [10] that there is a leghaemoglobin gene which, in addition to being expressed in nodules, is induced in plant cells containing arbuscules induced during the symbiosis with a mycorrhizal fungus. The role of this leghaemoglobin during mycorrhization is certainly not related to symbiotic nitrogen fixation, and its function can currently only be guessed at. The plants produced by Ott *et al*. [2] should enable them to test how important expression of leghaemoglobins will be during establishment of mycorrhizal symbioses.

The presence of other haemoglobins in plants might shed some light on possible alternative functions for leghaemoglobins. In addition to the legume symbiotic leghaemoglobins, most plants (including legumes) have other non-symbiotic haemoglobin genes [11–13]. The role of these non-symbiotic genes remains obscure, because it has been shown that their oxygen binding

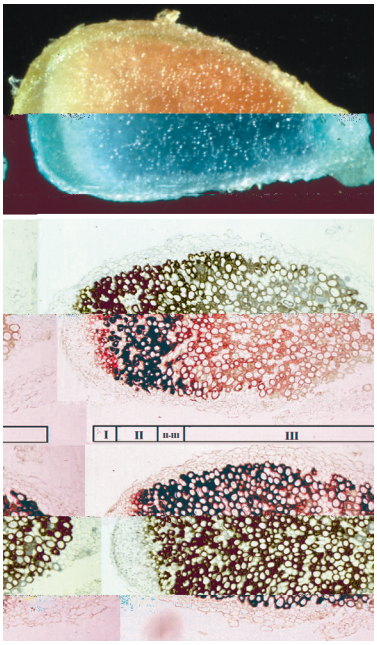

Figure 1. Longitudinal sections of pea nodules showing leghaemoglobin expression.

Top: photograph of a pea nodule cut in half showing the red colour of leghaemoglobin. Middle: section of a similar nodule, showing the pattern of expression of RNA encoding a leghaemoglobin (*PsLb120-1*) with a lower affinity for oxygen. Bottom: same, except that the probe detected the expression of RNA encoding a leghaemoglobin (*PsLb5-10*) with a higher affinity for oxygen. The expression patterns in the lower two panels were revealed using digoxigenin-labelled antisense probes [6]. The bars indicate the different zones of the nodule: I the meristem; II the infection zone; II-III the interzone where bacteroid differentiation occurs; III the nitrogen fixation zone. (Top image courtesy of N. Brewin; bottom images courtesy of N. Suganuma and H. Kouchi, reproduced with permission from [6].)

characteristics and cytoplasmic concentrations are not appropriate for them to act as oxygen carriers or an oxygen buffer; they simply bind oxygen too tightly [11–14].

Surprisingly these plant haemoglobins show some structural similarities to the recently discovered human haemoglobins, neuroglobin and histoglobin [3]. It has been proposed that this subclass of plant and animal haemoglobins may have a common function that could be related to disease resistance and stress pathways that induce production of nitric oxide and reactive oxygen species [3,15]. All haemoglobins can bind