Nutrient Sharing between Symbionts

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In this review, we consider the exchange of nutrients between the host plant and the bacterial microsymbiont in nitrogen-fixing legume root nodules. During nodule formation, the host tissues and the bacterial microsymbiont develop in response to each other to form a specialized tissue that maintains an environment where nitrogen fixation can occur (Brewin, 2004; Mergaert et al., 2006; Prell and Poole, 2006). This complex development will not be considered here but, at the end of the process, specialized, nitrogen-fixing forms of the bacteria, known as bacteroids, reside in the plant cytosol, enclosed within plant-derived membranes. These organelle-like structures are known as symbiosomes; the plant-derived membrane that surrounds the bacteroid is the symbiosome (or peribacteroid) membrane and the space between the two is the symbiosome (or peribacteroid) space. An infected plant cell may be packed with thousands of symbiosomes. The exchange of nutrients that is fundamental to N_2 fixation therefore involves metabolism in the plant to provide carbon and nitrogen compounds to the bacteroids and to assimilate the metabolites that bacteroids release. Nutrients transferred between the symbionts must traverse both the symbiosome and the bacteroid membranes. It is clear that there is more than one pattern whereby successful nutrient exchange can take place: There are two basic types of legume nodules, determinate and indeterminate, and there are fundamental differences between the two in how they develop and in their carbon and nitrogen metabolism. This review focuses on the metabolism of carbon and nitrogen compounds in the symbionts and on the exchange of nutrients across the bacteroid and symbiosome membranes. Particular attention is paid to the movement of primary carbon and nitrogen sources and how they are utilized by both bacteroids and the plant.

SUCROSE TRANSPORT AND CARBON PROVISION TO THE BACTEROID

A mature legume nodule must provide the proper chemical environment for the reduction of N_2 by bacteroids to ammonium. All the carbon and nitrogen sources as well as ions and oxygen must cross the symbiosome and bacteroid membranes, making them crucial to the establishment and maintenance of symbiosis (Fig. 1). Bacteroid metabolism should be considered similar to that of an organelle, since it is no longer a free-growing cell; instead it depends on the plant cell for all compounds. Bacteroid respiration requires a high flux of O_2 but this must be achieved while maintaining a very low concentration of free oxygen. Typically legume nodules maintain an O_2 concentration of around 3 to 22 nM (Witty, 1991; Hunt and Layzell, 1993) by the use of leghemoglobin, which consists of a family of O_2-binding heme proteins similar in function to myoglobin (Ott et al., 2005). A low oxygen environment is essential because the enzyme that reduces N_2, nitrogenase, is very oxygen sensitive. Bacteroid nitrogenase catalyzes the six-electron reduction of N_2 to ammonium and has an associated reduction of $2H^+$ to H_2 that uses 16 to 18 molecules of ATP (Dixon and Kahn, 2004). Ultimately, the symbiosis is driven by the plant, providing a carbon source to the bacteroid, the metabolism of which fuels N_2 fixation in exchange for secretion of a fixed nitrogen source. This is an energetically very expensive process and explains why nodulation is inhibited by the presence of fixed nitrogen, such as nitrate. This review is structured such that we first consider how the plant provides photosynthate, as Suc, to the nodule and how this is catabolized. We then address how this carbon source, as well as other essential nutrients, are transported to the bacteroid and metabolized to provide energy for nitrogen fixation. Finally, the process by which the product of nitrogen fixation, ammonium, is exported back to the plant and is assimilated into either amino acids or ureides for export to the shoot is considered.

The carbon supply, required to fuel nitrogenase activity in the bacteroid, is derived from plant photosynthate that is transported to the nodules via the phloem as Suc (Gordon et al., 1999). Proteomic and transcriptional analysis has revealed the presence of several sugar transporters expressed at the symbiosome membrane of *Lotus japonicus* and *Medicago truncatula* (Colebatch et al., 2002; Wienkoop and Saalbach, 2003; El Yahyaoui et al., 2004; Kouchi et al., 2004). However, transport of sugars has only been demonstrated across the symbiosome membrane of *Phaseolus* beans (*Phaseolus vulgaris*), and in other plant nodules it appears transport only occurs via diffusion, for which

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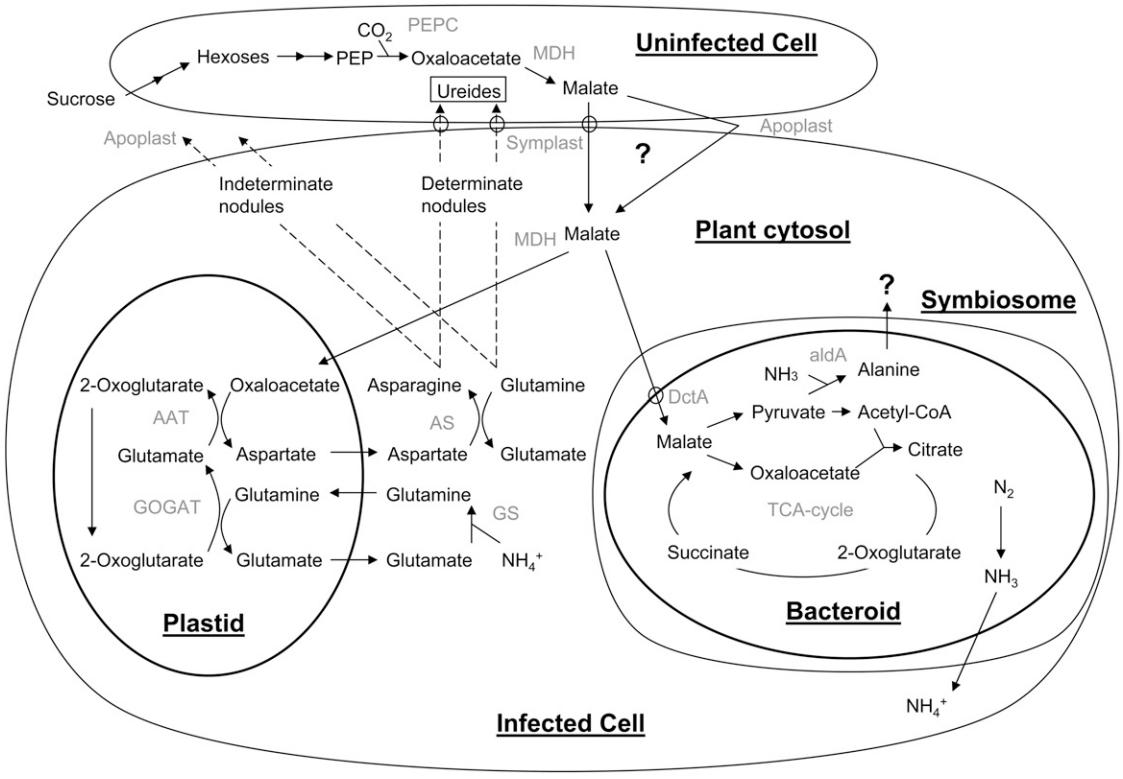

Figure 1. Proposed nutrient exchanges in legume nodules. In determinate nodules there are usually several bacteroids enclosed by a symbiosome membrane unlike the single bacteroid typical of indeterminate nodules. Each infected cell in a determinate nodule is usually in contact with an uninfected cell while infected cells of indeterminate nodules rarely are. Ureide synthesis only occurs in uninfected cells of determinate nodules.

the rate of uptake is not sufficient to support nitrogen fixation (Herrada et al., 1989; Udvardi and Day, 1997; Day et al., 2001). This reinforces the widely accepted view that dicarboxylic acids and not sugars are supplied to bacteroids.

Nitrogen-fixing bacteroids are located in plant cells in the center of the nodule, whereas the phloem is located within the nodules vascular network system in the inner cortex and is enclosed within an epidermis that serves as an apoplastic barrier (Abd-Alla et al., 2000; Hartmann et al., 2002). Supply of carbon to infected cells may require symplastic transport from uninfected cells, since uninfected, but not infected, cells of *Vicia faba* are able to actively take up Glc or Suc from the apoplast (Peiter and Schubert, 2003). Consistent with this a Suc/H^+ cotransporter (*Lj*SUT4) from *L. japonicus* nodules has been characterized and expression of this transporter in mature nodules was restricted mainly to the vascular bundles and nodule parenchymatous cells, while no hybridization signal could be detected in the cells of the central tissue (Flemetakis et al., 2003; Colebatch et al., 2004). Taken together with the expression of Suc synthase transcripts from soybean (*Glycine max*) nodules, this indicates that uninfected cells actively accumulate sugars and convert these to organic acids, which may then be released to the apoplast (Kavroulakis et al., 2000). Organic anions could then either be passively accumulated into the cytosol of infected cells due to the low apoplastic pH or be transferred symplastically from uninfected cells. Organic ions, almost certainly as dicarboxylic acids, are then supplied to bacteroids as the carbon source for metabolism and nitrogen fixation. Further evidence that Suc metabolism probably occurs in the uninfected cells of the nodule cortex, is that the low oxygen tension in infected cells prevents mitochondrial respiration from supplying carbon to bacteroids at a sufficient rate for sustained nitrogen fixation (Day and Copeland, 1991).

Suc can be cleaved either by Suc synthase, to produce UDP-Glc and Fru, or alkaline invertase, and the activity of both enzymes is higher in uninfected cells of the nodule than those of the surrounding roots (Singh et al., 1994; Chopra et al., 1998; Gordon et al., 1999; Flemetakis et al., 2006). Evidence for a nodule-enhanced Suc synthase has been demonstrated in *V. faba*, *M. truncatula*, and *P. vulgaris* (Kuster et al., 1993; Perlick and Puhler, 1993; Hohnjec et al., 1999; Silvente et al., 2002). In addition, Suc synthase mutants (*rug4*) of pea (*Pisum sativum*), which lack enzyme activity, are unable to produce nitrogen-fixing nodules, indicating that the enzyme is essential for nitrogen

fixation (Gordon et al., 1999). Interestingly, peas mutated in *rug4* contain *Rhizobium leguminosarum* bacteroids that are fully formed and possess nitrogenase protein but there is no effective N_2 reduction (Craig et al., 1999). This is consistent with the plant failing to provide carbon and energy to the bacteroid for N_2 reduction. While an absolute requirement for Suc synthase in pea nodules is well defined, it has been proposed that alkaline/neutral invertase is important for releasing hexoses and starch production in *L. japonicus* nodules (Flemetakis et al., 2006). The activity of both alkaline/neutral invertase and Suc synthase are elevated in *L. japonicus* nodules and the transcripts for Suc synthase and alkaline/neutral invertase (*LjInv1*) are elevated in both infected and uninfected cells (Flemetakis et al., 2006). Suc synthase in soybean (nodulin 100) can exist both in the cytoplasm and associated with the plasma membrane (Zhang et al., 1999). Both the plasma membrane-associated and soluble forms of the enzyme contain an N-terminal Ser (Ser-11) that can be phosphorylated in response to changes in stress (Komina et al., 2002). The phosphorylation state and the total protein content for both forms of the enzyme decreased upon the addition of NaCl or NH_4Cl stress, suggesting that the dephosphorylated form may be a better target for proteolysis.

The hydrolyzed products of Suc metabolism are used either for cellulose and starch biosynthesis, or further metabolized by glycolytic enzymes to produce phosphoenolpyruvate (PEP), which can be carboxylated to oxaloacetate and then reduced to malate for supply to the bacteroid (Rosendahl et al., 1990; Day and Copeland, 1991). Recent metabolomic analysis has confirmed the increased concentration of glycolytic intermediates and the synthesis of large amounts of organic acids in alfalfa (*Medicago sativa*) nodules relative to roots (Barsch et al., 2006b). Glycolysis is enhanced in nodules compared to roots and the relative abundance of Fru-6-P and Glc-6-P is also 5-fold higher in the nodules of *L. japonicus*, while the concentrations of Fru and Glc are much lower (Day and Copeland, 1991; Desbrosses et al., 2005). It has been shown that the transcripts for plastid-localized isozymes of hexokinase, phosphoglucomutase, and phospho-Glc isomerase are up-regulated in nodules of *L. japonicus*, while the cytosolic forms are similar to those in uninfected roots (Flemetakis et al., 2006). The activity of PEP carboxylase in nodules of alfalfa, soybean, mung bean (*Vigna radiata*), and lentil (*Lens culinaris*) is higher than that in surrounding roots, indicating that it plays a crucial role in providing carbon skeletons to infected cells for both effective nitrogen assimilation and also bacteroid metabolism to fuel nitrogenase activity (Miller et al., 1987; Pathirana et al., 1992; Vance and Gantt, 1992; Chopra et al., 2002). PEP carboxylase activity in soybean nodules is activated by a PEP carboxylase kinase that phosphorylates it on a Ser residue, particularly in response to the supply of photosynthate (Zhang et al., 1995; Wadham et al., 1996; Zhang and Chollet, 1997). For example, when plants are stem girdled or darkened, the phosphorylation and activity of PEP carboxylase declines. A decrease in phosphorylation of PEP carboxylase also makes the enzyme more sensitive to malate inhibition (Schuller et al., 1990; Schuller and Werner, 1993; Zhang et al., 1995). Thus in active legume nodules the enzyme is probably highly phosphorylated and insensitive to malate inhibition, ensuring continued synthesis of malate. *L. japonicus* and soybean have nodule-enhanced isoforms of PEP carboxylase (*LjPEPC1* and *GmPEPC7*, respectively) and PEP carboxylase kinase (*LjPEPC-PK* and *NE-PpcK*, respectively), further supporting their role in controlling the carbon supply to bacteroids (Hata et al., 1998; Nakagawa et al., 2003; Xu et al., 2003). The transcript levels of both PEP carboxylase and the PEP carboxylase kinase in soybean and *L. japonicum* change in the same way that enzyme activity does in response to the supply of photosynthate (Nakagawa et al., 2003; Xu et al., 2003). Decreasing the level of expression of *Ljpepc1* significantly lowered the enzyme activity of Suc synthase and also limited nitrogen fixation (Nomura et al., 2006).

The product of PEP carboxylase is oxaloacetate and this substrate is used by malate dehydrogenase to produce malate for supply to the bacteroid. Proteomic and transcriptional analysis of pea, *L. japonicus*, and *M. truncatula* nodules have shown that malate dehydrogenase and PEP carboxylase are up-regulated (Colebatch et al., 2002; Saalbach et al., 2002; Wienkoop and Saalbach, 2003; Colebatch et al., 2004; El Yahyaoui et al., 2004; Kouchi et al., 2004). Furthermore, the expression of these genes is reduced in ineffective nodules with defective nitrogenase activity (Haser et al., 1992; Vance et al., 1994; Suganuma et al., 2004). Malate dehydrogenase activity in nodules is rapidly enhanced as bacteroids develop and this is also linked to an increase in malate concentration (Appels and Haaker, 1988; Ratajczak et al., 1989; Colebatch et al., 2004). A nodule-enhanced malate dehydrogenase has been detected in nodules of pea and alfalfa, where its transcript is more highly expressed. However, its subcellular location in nodule plant cells remains unknown (Appels and Haaker, 1988; Miller et al., 1998; Fedorova et al., 1999b). Five different forms of malate dehydrogenase were cloned from alfalfa and of these the nodule-enhanced form (neMDH) made up 50% of the enzyme in nodules (Miller et al., 1998). The K_m values for oxaloacetate and NADH are also significantly lower than for malate and NAD^+, indicating malate synthesis is favored.

A number of early studies showed that dicarboxylates stimulate bacteroid nitrogen fixation in vitro, indicating their role as the carbon source for bacteroid metabolism in planta (Poole and Allaway, 2000; Lodwig and Poole, 2003). The concentrations of dicarboxylates in nodules are high, and labeling experiments with $^{14}CO_2$ demonstrated a high turnover of these pools as the label was rapidly incorporated into the bacteroids, primarily as malate (Rosendahl et al., 1990; Salminen and Streeter, 1992). While transport of sugars has only been demonstrated across the symbiosome membrane

of *P. vulgaris* nodules, the transport of dicarboxylates at high rates has been demonstrated across the symbiosome membrane of all nodules without exception, indicating their role as the principal carbon source for bacteroid metabolism (Herrada et al., 1989; Ouyang et al., 1990; Ouyang and Day, 1992). Consistent with this the C_4 dicarboxylate transport system (Dct) has been shown to be essential for N_2 fixation by bacteroids of white clover (*Trifolium repens*), peas, alfalfa, and soybean (Poole and Allaway, 2000; Lodwig and Poole, 2003; Yurgel and Kahn, 2004).

TRANSPORT ACROSS THE SYMBIOSOME AND BACTEROID MEMBRANES

It is important to appreciate that nutrient exchange between bacteroids and the plant cytosol requires transport across both the plant-derived symbiosome membrane and the bacteroid membrane. Since the symbiosome membrane is derived from endocytosis of bacteroids by the plant plasma membrane, it is inverted and therefore solute movement from the plant cell to the symbiosome space resembles export. Transport from the symbiosome space into the bacteroid is similar to uptake in free-living bacteria.

Most studies on transport with symbiosomes have been done with symbiosomes from determinate nodules, such as soybean, because the symbiosome membrane encloses several bacteroids facilitating their separation from unenveloped free bacteroids on density gradients (Day et al., 2001; Lodwig and Poole, 2003). C_4 dicarboxylates are transported at high rates, consistent with their role as the principal carbon source supplied to bacteroids. Isolated soybean symbiosome units are impermeable to the active movement of sugars (Suc, Fru, and Glc) and amino acids from the plant cytosol to the bacteroid (Udvardi and Day 1997; Day et al., 2001; Lodwig and Poole, 2003). A significant caveat must be applied to all studies of symbiosomes since the isolation of what is effectively a fragile intracellular structure may result in the loss of factors needed by some transport systems. Likewise, the isolated membrane may not be energized properly, all of which would result in the selective loss of detectable uptake activity.

The identity of the dicarboxylate transporter on the symbiosome membrane is still unknown. Nodulin 26 was purified from soybean symbiosome membranes and reconstituted into planar lipid bilayers and shown to reconstitute an ion channel with weak selectivity for anions (Shomer et al., 1994; Weaver et al., 1994). However, it has now been shown to be an aquaporin that transports water and glycerol (Dean et al., 1999). More recently a cDNA has been isolated that codes for a dicarboxylate transporter (AgDCAT1), which is a member of the peptide transporter family, and has been specifically located in the symbiotic membrane of Alder plants (Jeong et al., 2004). Alders form a N_2-fixing symbiosis with Frankia, a filamentous actinomycete, and AgDCAT1 actively transports the dicarboxylates, malate, succinate, fumarate, and oxaloacetate (K_m for malate 70 μM). While the legume dicarboxylate transporter, located in symbiosome membranes, may not be related to this system, this is none the less an exciting development.

As previously discussed, numerous earlier studies showed that C_4 dicarboxylates are required by the bacteroid for nitrogen fixation and mutants of the Dct system cause a Fix^- phenotype on plants (Yurgel and Kahn, 2004). The system is made up of three genes: *dctA* encoding a transport protein, and *dctB* and *dctD*, which are divergently transcribed and encode a two-component sensor regulator system that activates transcription of *dctA* in response to C_4 dicarboxylates (Watson, 1990; Reid and Poole, 1998; Yurgel et al., 2000; Yurgel and Kahn, 2005). DctA of *Rhizobium* strains is typical of most bacterial DctA carriers in being a cation (H^+) symporter of around 440 amino acids (Janausch et al., 2002). DctA was thought to have 12 putative transmembrane α-helices (Jording and Pühler, 1993), with the N and C termini located in the cytoplasm, but it has more recently been proposed that members of the Glu transporter family, which includes DctA, have 10 membrane-spanning α-helices (Slotboom et al., 1999). Early studies using clover and pea bacteroids suggested that *dctA* mutants formed fully developed bacteroids (Finan et al., 1981; Ronson et al., 1981). However, more recent studies with alfalfa bacteroids have shown severe developmental defects in *dctA* bacteroids (Yurgel and Kahn, 2004). To clear up this apparent contradiction a *dctA* mutant of *R. leguminosarum* bv *viciae* was inoculated onto pea plants and it can be seen that *dctA* bacteroids are severely impaired for development (Fig. 2).

NITROGEN FIXATION AND BACTEROID METABOLISM OF DICARBOXYLATES

The tricarboxylic acid (TCA) cycle is the central metabolic pathway in rhizobia and C_4 dicarboxylic acid metabolism is required to drive nitrogen fixation (Lodwig and Poole, 2003). Oxidation of compounds via the TCA cycle provides reducing equivalents, ATP synthesis, and metabolites for amino acid production and other biosynthetic pathways. However, it is surprising that mutational analysis suggests a full TCA cycle may not be essential for N_2 fixation in slow-growing rhizobia, such as *Bradyrhizobium japonicum*, that form determinate nodules. By contrast, the evidence so far indicates that the faster-growing rhizobia, such as *Sinorhizobium meliloti* and *R. leguminosarum*, do need a complete TCA cycle. The first indication that a full TCA cycle may not operate in bacteroids was the demonstration that aconitase mutants of *B. japonicum* can still establish effective nitrogen fixation with soybean plants (Thonymeyer and Kunzler, 1996). Aconitase catalyzes the reversible isomerization of citrate and isocitrate, however, mutation in *acnA* only abolished

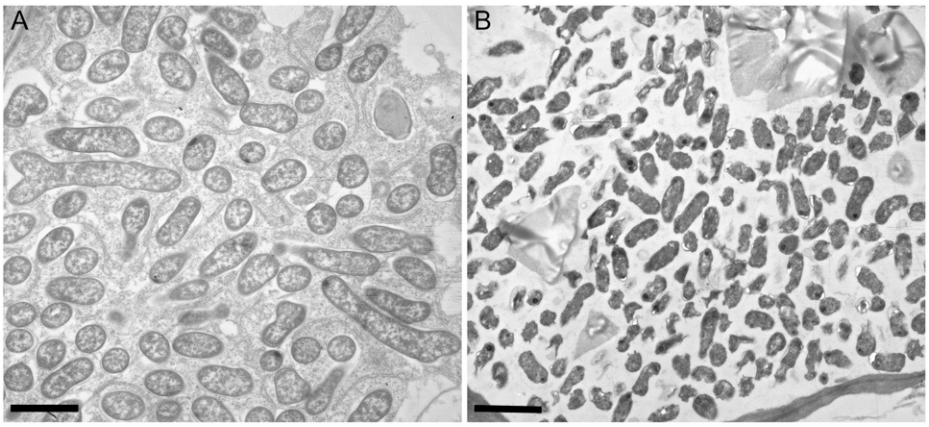

Figure 2. Electron micrographs of 21-d-old pea nodules. A, *R. leguminosarum* bv *viciae* 3841. B, RU437 (3841 *dctA*::Tn5; Poole et al., 1994); bar = 2 μm.

70% of the activity in free-living cells, suggesting the presence of a second activity.

Isocitrate dehydrogenase mutants of *B. japonicum* are only slightly delayed in nodule formation on soybean and fix nitrogen at similar rates to wild type (Shah and Emerich, 2006), while *S. meliloti* isocitrate dehydrogenase mutants are ineffective, although they form normal nodules full of bacteroids (McDermott and Kahn, 1992). This emphasizes the difference between soybean nodulating bacteria and those that nodulate temperate legumes such as alfalfa and pea.

The *sucAB* genes encode the 2-oxoglutarate dehydrogenase component (E1) and dihydrolipoamide succinyl transferase component (E2) of the 2-oxoglutarate dehydrogenase complex, respectively. These catalyze the oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA (Green and Emerich, 1997a; Walshaw et al., 1997). Nodulation of soybean plants by a *sucA* strain of *B. japonicum*, while significantly delayed and reduced, did produce bacteroids capable of nitrogen fixation at rates comparable to wild type when expressed per cell (Green and Emerich, 1997b). By contrast a *sucA* mutant of *R. leguminosarum* did not fix N_2 on peas (Walshaw et al., 1997). Thus, just as for isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase mutants behave differently between *B. japonicum* and rhizobia that nodulate temperate legumes.

Taken as a whole, the experiments with TCA-cycle mutants of *B. japonicum* suggest it is possible to block the TCA cycle in this organism and still permit N_2 fixation in soybean nodules. Of course mutational analysis is not the same as flux analysis and mutant bacteroids may be able to bypass at least part of the TCA cycle even though it may still be highly active in the wild type. An alternative pathway for 2-oxoglutarate metabolism in *B. japonicum*, which can be blocked by a *sucA* mutation and still be capable of fixing N_2, has been demonstrated through the activities of 2-oxoglutarate decarboxylase and succinate semialdehyde dehydrogenase (Green et al., 2000). Both enzymes are active in isolated bacteroids from soybean and suggest an alternative to the TCA cycle. Genes for a three-protein 2-oxoglutarate:acceptor oxidoreductase (KGOR) complex, which catalyzes the oxidative decarboxylation of 2-ketoglutarate to succinyl-CoA, with concomitant reduction of either $NADP^+$ or ferredoxin, has been identified in *Azoarcus evansii* (Ebenau-Jehle et al., 2003). It was also proposed that a similar *B. japonicum* 2-oxoacid oxidoreductase (BjOOR) complex is present in *B. japonicum*. This complex is made up of blr6742, blr6743, and blr6744 and may be the 2-oxoglutarate decarboxylase complex identified by Green et al. (2000). Curiously, good candidates for all of these genes are not present in *R. leguminosarum* 3841 or *Mesorhizobium loti* R7A, even though these strains also showed 2-oxoglutarate decarboxylase activity in the same study.

The first step of the TCA cycle is the synthesis of citrate resulting from the condensation of oxaloacetate and acetyl-CoA. It is clear that oxaloacetate is derived from malate, succinate, and fumarate provided by the plant but acetyl-CoA is thought to be derived via a two-step process. Malate is oxidatively decarboxylated by malic enzyme to pyruvate and this is converted to acetyl-CoA by pyruvate dehydrogenase (McKay et al., 1988; Finan et al., 1991; Driscoll and Finan, 1993; Driscoll and Finan, 1996; Cabanes et al., 2000; Mitsch et al., 2007). Pyruvate dehydrogenase is a multienzyme complex encoded by two genes, *pdhA*α and *pdhA*β, in *S. meliloti* (Cabanes et al., 2000). Mutation of a putative arylesterase (*ada*), that clusters with the genes for pyruvate dehydrogenase, reduced the activity of pyruvate dehydrogenase in *S. meliloti* 16-fold and resulted in a Fix^- phenotype (Soto et al., 2001).

In rhizobia there are two forms of malic enzyme: an $NADP^+$-dependant malic enzyme with high affinity that is stimulated by ammonium, and an NAD^+-dependant malic enzyme with a lower affinity that is stimulated by potassium and ammonium salts (Finan et al., 1991; Driscoll and Finan, 1993; Driscoll and Finan, 1996). NAD^+-malic enzyme is essential for nitrogen fixation in alfalfa and is highly expressed in both free-living cells and bacteroids. $NADP^+$-malic enzyme is dispensable for nitrogen fixation and is repressed in bacteroids (Driscoll and Finan, 1997; Djordjevic, 2004; Sarma and Emerich, 2005; Mitsch et al., 2007). Placing $NADP^+$-dependant malic enzyme

under the control of the NAD^+-dependent malic enzyme promoter, which dramatically increases its expression and enzyme activity, did not enable it to substitute for the NAD^+-dependent activity in N_2 fixation (Mitsch et al., 2007). This suggests that $NADP^+$ cannot replace NAD^+, perhaps because high levels of NADPH prevent reduction of $NADP^+$.

EXPORT OF NITROGEN TO THE PLANT AND ITS ASSIMILATION

Classical labeling studies had shown that the ammonium derived from the reduction of N_2 by bacteroid nitrogenase is directly exported from the plant, where it is assimilated into amino acids (for review, see Lodwig and Poole, 2003). In a recent report using $^{15}N_2$ labeling of soybeans it was reported that Ala rather than ammonium is the sole secretion product (Waters et al., 1998). However, others failed to repeat this experiment under similar conditions using the same bacterial strains inoculated onto soybean (Li et al., 2002). In a direct attempt to address this contradiction, pea plants were incubated in $^{15}N_2$ and found to secrete predominantly ammonium but also significant amounts of Ala (Allaway et al., 2000), with the ratio depending on the concentration of ammonium that had built up in the medium. This is understandable because the principal way of making Ala in bacteroids is via the reductive amination of pyruvate by Ala dehydrogenase. This enzyme has a K_m for ammonium of 5 mM (Allaway et al., 2000), explaining why ammonium must be at millimolar concentrations to enable Ala secretion. Indeed, when Ala dehydrogenase (*aldA*) was mutated in pea bacteroids only ammonium and not Ala was secreted (Allaway et al., 2000). Most importantly, when AldA enzyme activity was removed by mutation of the genes in either *R. leguminosarum* or *M. loti*, which has two actively expressed *aldA* genes, there was no alteration in N_2 fixation in nodules of pea (indeterminate nodules) or *Lotus corniculatus* (determinate nodules; Allaway et al., 2000; Kumar et al., 2005). Given the demonstration by $^{15}N_2$ labeling that Ala is synthesized by AldA, this makes it highly improbable that Ala could be the sole nitrogen export product in pea or *L. corniculatus*. While the results for soybean are disputed, the overall evidence suggests it is unlikely that Ala is the sole secretion product in planta, even if under some circumstances in the laboratory isolated bacteroids only secrete Ala. To change this position, and accept that bacteroids from soybean differ from those in pea and *L. corniculatus*, we believe that the double *aldA* mutant would need to be made in *B. japonicum*. Furthermore, this *B. japonicum* mutant when inoculated onto soybean would have to be severely perturbed in nitrogen fixation, unlike the situation for *R. leguminosarum* and *M. loti* inoculated onto pea and *L. corniculatus*, respectively.

Ammonium transport by free-living rhizobia grown under nitrogen limitation occurs through active uptake via the Ntr-regulated Amt transporters (Day et al., 2001). During symbiosis the Amt is not expressed, in fact ectopic expression of this system in *Rhizobium etli* disrupts nodulation and bacteroid differentiation (Tate et al., 1998, 1999). Therefore, it is expected that diffusion of ammonia occurs from nitrogen-fixing bacteroids into an acidified symbiosome space to enhance NH_4^+ formation for transport across the symbiosome membrane (Tyerman et al., 1995; Day et al., 2001). It was also proposed that nitrogen efflux from the symbiosome space to the plant cytosol occurs via diffusion because a large concentration gradient of ammonia exists (Udvardi and Day, 1990). However, a voltage-gated nonselective cation channel capable of NH_4^+ transport has been identified on the symbiosome membrane from soybean, pea, and *L. japonicus* that will allow transport when a positive membrane potential is generated across the symbiosome membrane (Tyerman et al., 1995; Mouritzen and Rosendahl, 1997; Kaiser et al., 1998; Roberts and Tyerman, 2002). These channels are typically inwardly rectified by the presence of Ca^{2+} or Mg^{2+}, and it was proposed that the direction of ammonium movement is from the symbiosome space to the plant cytosol with cytosolic Mg^{2+} being the main regulator (Roberts and Tyerman, 2002). However, there is also kinetic evidence that ammonia gas (NH_3) may diffuse through a channel on the symbiosome membrane (Niemietz and Tyerman, 2000).

Acidification of the symbiosome space would generate a potential, energizing the membrane to allow movement of NH_4^+ into the plant cytosol. The acidification of the symbiosome space is facilitated by a proton pumping H^+-ATPase (Blumwald et al., 1985; Udvardi and Day, 1989; Szafran and Haaker, 1995). Biochemical and immunological studies revealed that the major H^+-ATPase of symbiosis is a P-ATPase (Blumwald et al., 1985; Fedorova et al., 1999a). Proteomic studies of the symbiosome membrane fraction of *L. japonicus* and pea have also revealed the presence of V-type H^+-ATPases (Saalbach et al., 2002; Wienkoop and Saalbach, 2003).

While considering ammonium movement in legume nodules it is important to appreciate that the entire ionic environment of bacteroids will be determined by the symbiosome membrane. This has stimulated a number of studies of specific ion transporters. For example, a symbiosome membrane-specific sulfate transporter (SST1) has been mutated and cloned in *L. japonicus* and shown to be essential for N_2 fixation (Krusell et al., 2005). Similarly, a nodule-enhanced transporter for ferrous iron (GmDmt1), belonging to the natural resistance-associated macrophage protein family has been identified in soybean (Kaiser et al., 2003). Such a system may be important for provision of iron to the bacteroid, and therefore essential to nitrogen fixation. Inorganic ion transporters (nitrate, nitrite, and chloride) in *L. japonicus* (LjN70; (Szczyglowski et al., 1998) and soybean (GmN70; Vincill et al., 2005) have also been localized to the symbiosome membrane as has a zinc transporter (GmZip1) in soybean (Moreau et al., 2002).

The ammonium released by bacteroids is assimilated into Gln via the Gln synthetase/Glu synthase (GS/GOGAT) pathway in plant cells (Patriarca et al., 2002; Barsch et al., 2006a). Temperate legumes that tend to form indeterminate nodules, such as pea, clover, and alfalfa, mainly export Asn out of the nodule to the shoot, whereas tropical legumes that tend to form determinate nodules, such as soybean and *Phaseolus* bean, export ureides (Temple et al., 1998; Goggin et al., 2003). Alfalfa nodules have three types of GS, two cytosolic isoenzymes of GS1 (GS13 and GS100) and a plastid enzyme, GS2 (Temple et al., 1996, 1998). Of these, GS13 is nodule enhanced. Transcription of the gene encoding NADH-dependant GOGAT and enzyme activity, which is plastid localized, is also induced in infected cells compared to those of uninfected cells and other plant organs (Vance and Gantt, 1992; Vance et al., 1995; Trepp et al., 1999a). An expression map for these genes, as well as several required for carbon metabolism in the plant cytosol and bacteroid, has been derived for alfalfa nodules (Trepp et al., 1999b). This shows that N_2 fixation and assimilation is most active in a five to 15 cell-wide zone in the distal part of zone III (close to the II/III interzone). Transcriptome, proteomic, and metabolite profiling by gas chromatography-mass spectrometry of *L. japonicus* supported the biosynthesis of Gln and Asn and identified induction of two genes encoding GS enzymes and two genes encoding Asn synthetases (Wienkoop and Saalbach, 2003; Colebatch et al., 2004; Desbrosses et al., 2005). Consistent with the assimilation of ammonium by the plant, antisense inhibition of NADH GOGAT in alfalfa caused severe inhibition of nitrogen fixation, with the formation of chlorotic plants (Cordoba et al., 2003). Finally, in most temperate legumes the assimilated Gln is exported out of the nodule, principally as Asn, which requires the concerted activity of a Gln-dependent Asn synthetase and Asp aminotransferase. In alfalfa, there is a nodule-enhanced Gln-dependent Asn synthetase (Shi et al., 1997) and in *L. corniculatus* and alfalfa there are nodule-enhanced Asp aminotransferases (P2 and AAT2, respectively), which at least in alfalfa has been located in a plastid (Gregerson et al., 1994; Mett et al., 1996). In indeterminate *V. faba* nodules most infected cells do not make contact with uninfected cells and it has been proposed that amino acids are released by infected cells into the apoplast from which they can be actively accumulated by uninfected cells (Peiter et al., 2004). Uninfected cells could then symplastically transfer amino acids to the vascular system (Abd-Alla et al., 2000; Peiter et al., 2004). In determinate soybean nodules, all infected cells appear to have contact with uninfected cells and ureides are synthesized by uninfected cells for export in the xylem (Schubert, 1986; Selker, 1988).

AMINO ACID CYCLING

Labeling studies with pea, soybean, and lupin bacteroids demonstrated that they secrete the amino acids Ala or Asp under N_2-fixing conditions (Kretovich et al., 1986; Appels and Haaker, 1991; Rosendahl et al., 1992; Waters et al., 1998; Allaway et al., 2000). One possibility is that instead of ammonium, Ala, or Asp are the primary secretion products of N_2 fixation (see above), but we consider the evidence so far makes this unlikely. Another possibility is that this is part of a malate-Asp shuttle (Kahn et al., 1985; Appels and Haaker, 1991), but the labeling studies have poor rates of keto acid uptake and secretion rates that are difficult to reconcile with a malate/Asp shuttle. However, mutation of the two broad range amino acid ATP-binding cassette-type transport systems (Aap and Bra) in *R. leguminosarum* had a dramatic impact on N_2 fixation. The plants were severely nitrogen starved even though peak $^{15}N_2$-fixation rates per plant were around 30% of wild-type levels and *aap/bra* mutants retained nitrogenase activity at rates per bacteroid that equaled or exceeded the wild type (Lodwig et al., 2003). The Aap system consists of a solute-binding protein (AapJ), two integral membrane proteins (AapQM), and an ATP-binding cassette (AapP; Walshaw and Poole, 1996). The Bra system has a solute-binding protein BraC, two integral membrane proteins (BraDE), and two ATP-binding cassettes (BraFG; Hosie et al., 2001). These two systems actively transport a broad range of L-amino acids but also appear capable of passive efflux. Thus an amino acid must either be transported or secreted for effective nitrogen fixation and assimilation to occur in pea nodules. It was suggested that an amino acid cycle might operate, where an amino acid such as Glu or possibly γ-amino butyric acid is taken up by bacteroids and used to transaminate either oxaloacetate or pyruvate for secretion of Asp or Ala, respectively (Lodwig et al., 2003; Prell and Poole, 2006). The Aap and Bra might be required for either uptake and/or efflux of the amino acids. There was no evidence that keto acids also cycle, as would occur in a true malate Asp shuttle, so this model was called an amino acid cycle. Consistent with such a cycle Asp aminotransferase (AatA) is essential for N_2 reduction in both pea and alfalfa bacteroids (Rastogi and Watson, 1991; Lodwig et al., 2003). The biggest problem in understanding the function of amino acid movement in pea nodules is that Aap and Bra have a very broad solute specificity and while they are active uptake systems they also promote passive efflux. To further understand their role it is essential to limit the solute specificity of the Aap and Bra to determine which amino acids they actually transport and whether uptake and/or efflux is required. Furthermore, while an amino acid cycle has been proposed it is not strictly possible to rule out that an amino acid only moves in or out of the bacteroid. Thus, Aap and Bra may be required for a two-way cycle that provides reductant for N_2 fixation or for the specific provision of an amino acid to the bacteroid that is essential for maintenance of symbiosis.

For amino acid cycling to occur, amino acids must cross the symbiosome membrane. Transport studies

with symbiosomes isolated from soybean, *P. vulgaris*, and *V. faba* have failed to identify active amino acid transport across the symbiosome membrane (Udvardi et al., 1988; Herrada et al., 1989; Ouyang and Day, 1992; Trinchant et al., 1994). Many amino acid transporters are proton coupled and operate as symporters. While the pH gradient across the symbiosome membrane is in the wrong direction for import into the symbiosome it is in the right direction for export. An H^+/Asp export system, which moves solutes from the symbiosome space to the plant cytosol, has been identified on pea symbiosome membranes (Rudbeck et al., 1999; Rosendahl et al., 2001). Although no transport system has yet been identified for amino acid transport into symbiosomes, the isolation techniques used may damage the symbiosome membrane, or the membrane may be incorrectly energized, abolishing transport in vitro (Day et al., 2001). It has also recently been shown that isolated pea bacteroids show almost no amino acid uptake, but this is likely to be due to membrane damage and the need to use high osmolarity isolation medium that reversibly inactivates ATP-binding cassette uptake systems (Fox et al., 2006).

SUMMARY

A great deal of progress in understanding nutrient exchange has been made recently by the use of transcriptomic and proteomic analysis of legume nodules (Fig. 1). It is clear that there is a highly regulated exchange of carbon and nitrogen sources and this process drives N_2 fixation. Dramatic progress has been made in identifying plant genes for transporters and enzymes essential for symbiosis. There is, however, a great deal to be understood about the basic biochemistry and physiology of nodule function. These are actually old-fashioned biochemical questions that are still very difficult to answer. However, this should serve as a challenge for future studies where a rigorous metabolomic analysis of nodule function, including flux analysis, needs to be performed. This will help establish whether pathways such as the TCA cycle are being bypassed in bacteroids and why amino acid movement is essential to symbiosis.

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