Chapter 3
Carbon Metabolism During Symbiotic Nitrogen Fixation

Emmanouil Flemetakis and Trevor L. Wang

3.1 Introduction

Legumes have the ability to grow in low nitrogen soils because of their capacity to incorporate atmospheric nitrogen into amino acids. This ability has been gained via the sequestering of the pre-existing mycorrhizal developmental pathway (Parniske 2008) and through the formation of a specialised organ, the root nodule, in which there is a symbiotic relationship with rhizobium bacteria of the soil. The plant supplies carbon (C) to the symbiotic partner, and in return, the bacterium (in modified form known as a bacteroid) fixes atmospheric nitrogen (N_2) to generate amino acids that are supplied to the plant. Legumes pay a price for this relationship. In seminal studies from Pate's laboratory (Pate and Herridge 1977; Pate et al. 1979a, b), it was reported that in the annual lupin (*Lupinus albus* L.) approximately 51 % of C from photosynthesis was sequestered by the roots and 4.0–6.5 g C utilised by the nodule to fix every gram of nitrogen (N). However, 34 % of the C supplied to the nodule returned to the shoot in the form of symbiotic nitrogen fixation (SNF) products. This large commitment of C being translocated to nodules was supported by later studies on the same species (Layzell et al. 1981) and in other species (see Gordon 1992), which gave values between 40 % and 50 %. In a study on *Trifolium repens* L. cv. Blanca (white clover), Gordon et al. (1987) showed that half of the 45 % processed by the nodule was respired. Here, we review the importance of some recent developments and key enzymes involved in the metabolic pathway of carbon to the bacteroid.

E. Flemetakis (✉)
Laboratory of Molecular Biology, Department of Agricultural Biotechnology, Agricultural University of Athens, Iera Odos 75, Athens 11855, Greece
e-mail: mflem@aua.gr

T.L. Wang
Metabolic Biology, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK
e-mail: trevor.wang@jic.ac.uk

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The metabolic pathway of C supplied from the leaves of a legume plant to its root nodules can be divided into three phases: the catabolism to hexoses of photosynthesis-derived sucrose entering infected cells, the metabolism of hexoses to phosphoenolpyruvate (PEP) via glycolysis and the conversion of PEP to dicarboxylic acids (DCA) for transport into bacteroids. The upregulation of genes during nodulation (nodule enhanced, NE) as exemplified by the nodulin genes (Verma et al. 1986) has often been used as prime facie evidence for the specific involvement of their products in SNF. However, as we shall see later, this is not always the case as it may reflect another facet of the nodulation process not associated with nodule function per se, for example, cell division. The use of the model legumes *Lotus japonicus* and *Medicago truncatula* has greatly enhanced our understanding of nodulation and C delivery since one can investigate enzymes in the pathway through the use of specific mutants. Moreover, large datasets for transcripts and metabolites (e.g. http://mtgea.noble.org/v2/) are now available together with mutants for several genes encoding enzymes of the pathway (e.g. Horst et al. 2007). By interrogating such databases, one can clearly identify that a large set of genes concerned with carbohydrate metabolism is altered in expression during nodulation, and this is a reflection of both metabolism (Fig. 3.1) and the creation of a nodule.

3.2 Sucrose Catabolism

When sucrose from the aerial parts of the plant is delivered via the phloem to the root nodule, there are two routes by which it can be utilised by a cell, both involving enzymatic cleavage of the disaccharide to its individual monomers. The two enzymes involved are sucrose synthase (SUS, EC 2.4.1.13) and invertase (INV, EC 3.2.1.26), each using a different mechanism. The former catalyses the cleavage of sucrose to UDP-glucose and fructose in a reversible reaction, whereas the latter generates the individual hexoses, glucose and fructose, in an irreversible reaction. It was not until mutants of SUS were isolated in *Pisum sativum* (pea; Wang et al. 1998; Craig et al. 1999) that it became clear that this enzyme was the only one essential for SNF and assimilation.

3.2.1 Sucrose Synthase

Sucrose is believed to move to the infected core of the nodule via the uninfected cells. This is where the highest content of SUS can be found in both *Glycine max* (soybean) and white clover (Gordon et al. 1992, 1995). The first defined plant mutants shown to affect N assimilation were isolated from a forward screen of pea seeds for a wrinkled phenotype (Wang et al. 1990, 1998). The mutants were at the *rug4* locus of pea, and their seeds had a lowered starch content, which generated the

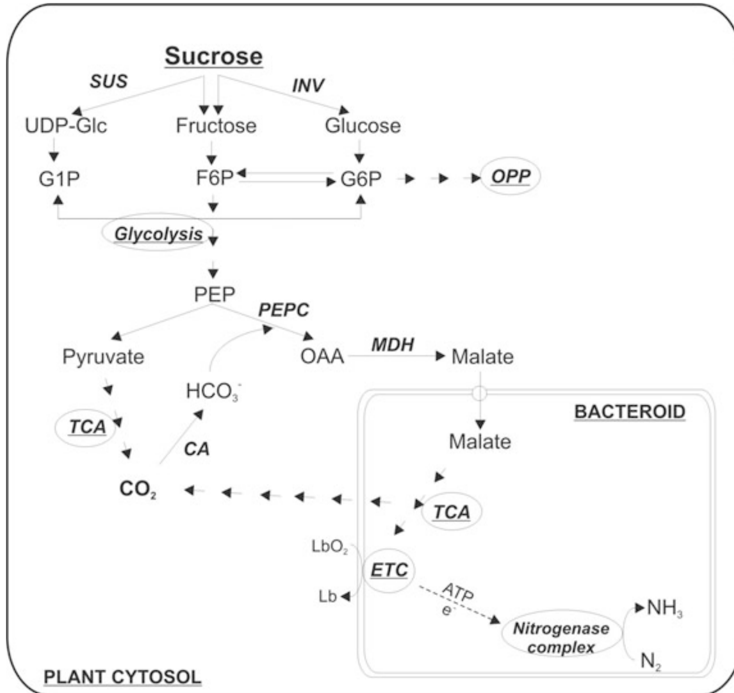

Fig. 3.1 Schematic representation of the possible sucrose catabolising metabolic pathways in nitrogen-fixing nodules. For simplicity, only enzymes discussed in this chapter are shown. *SUS* sucrose synthase, *INV* invertase, *PEPC* phospho*enol*pyruvate carboxylase, *CA* carbonic anhydrase, *MDH* malate dehydrogenase, *OPP* oxidative pentose phosphate pathway, *TCA* tricarboxylic acid cycle, *ETC* electron transport chain

wrinkled phenotype (Wang and Hedley 1991). Subsequently, it was found that the locus encoded a sucrose synthase (SUS) whose activity was clearly required for starch synthesis in the seed (Craig et al. 1999). The *rug4* mutant plants when grown in the presence of rhizobium in N-free composts or poor soil in the field showed severe symptoms of N starvation. The plants had low chlorophyll contents and a low N content indicating that they derived little of their N from fixation. Craig et al. (1999) concluded therefore that this SUS isoform was needed to supply C for SNF. Furthermore, the *rug4* nodules showed the early senescence phenotype that is characteristic of this class of mutants (e.g. Novak et al. 1995). Since the plants grew normally when supplied with N, it was concluded that normal growth and development was supported by other SUS isoforms. In a detailed analysis of these mutants, Gordon et al. (1999) showed that several changes occurred in the enzymes of N assimilation, some decreasing in activity (phosphofructokinase, PEP carboxylase, Gln synthase, Gln oxoglutarate aminotransferase and Asp aminotransferase), others increasing their activities (PPi-dependent, Fru-6-P phosphotransferase, pyruvate decarboxylase and alcohol dehydrogenase). INV also decreased and there was

substantially less leghaemoglobin in the mutants. Neither nodules nor isolated bacteroids of *rug4* mutants showed apparent nitrogenase activity despite the presence of normal levels of nitrogenase protein. Furthermore, mutant plants showed little of no N accumulation. Gordon et al. (1999) hypothesised that SUS was needed to maintain nitrogenase activity and they concluded that the *Rug4* isoform of SUS was essential for SNF, INV being unable to compensate for its loss in the mutant.

The *Rug4* isoform of SUS represents the NE form (nodulin-100; Thummler and Verma 1987). Studies by Barratt and co-workers showed that SUS was encoded by a small gene family in both pea (Barratt et al. 2001) and Arabidopsis (Bieniawska et al. 2007). In pea, there are six cytosolic isoforms of SUS, two of which are present in the nodule (SUS1 and SUS3; Barratt et al. 2001). It is in legumes where we see the most dramatic effect of reduced SUS activity, since a quadruple knockout mutant of the four main isoforms in Arabidopsis showed no phenotype indicating a high level of redundancy within the gene family in this species (Barratt et al. 2009). The use of *L. japonicus* permitted a further analysis of SUS isoforms. Like pea and Arabidopsis, there are six isoforms, and two are present in the nodule, LjSUS1 and LjSUS3 (Horst et al. 2007); LjSUS3 is the main and the NE isoform. However, when the NE isoform was knocked out through the isolation of a TILLING mutant creating a premature stop codon in the coding sequence, the plants could grow and fix nitrogen although with reduced capacity, the plants showing symptoms of N starvation. It was only when the activity of both isoforms was removed by creating a double mutant that the plant became severely impaired and unable to fix N, indicating that LjSUS1 must also contribute to the maintenance of N assimilation. The wild type (WT) phenotype could be restored, however, by application of N. The different contributions of the NE isoform in pea (*rug4*) and in Lotus (LjSUS3) to the total nodule SUS activity (90 % vs. 70 %) may account for the different phenotypes (Horst et al. 2007). In *M. truncatula*, when the gene encoding the main nodule-enhanced isoform (Hohnjec et al. 1999) was targeted by using an antisense construct (Baier et al. 2007), a severe decrease in protein content of up to 90 % in some transgenic lines was observed using western blotting. The *M. truncatula* downregulated plants showed a similar growth and development phenotype to those in the pea *rug4* and *L. japonicus* double mutants albeit less severe. However, enzyme activity was not measured in the *M. truncatula* plants and so it was not clear how much activity was present due to the remaining protein. Only the NE isoform was detected in nodules by western blotting, but an additional isoform was observed in roots. Interestingly, the antisense construct decreased the transcript and protein levels of *both* isoforms.

One further point can be made concerning the role of starch accumulation in nodule function from the detailed analysis of the *rug4* mutant by Gordon et al. (1999). The mutant nodules contained substantial amounts of starch albeit lower than in the WT, which indicates that nodule starch cannot be mobilised to substitute for the loss of UDP-glucose delivered by SUS. This is also supported by the fact that the starchless plastidial phosphoglucomutase mutants, *Psrug3* and *Ljpgm1,* have perfectly functional nodules. In contrast, *Ljgwd1* mutants that cannot break down starch and accumulate very large amounts in all starch-storing organs,

including nodules, have impaired nodule function (Vriet et al. 2010). Put together, these data indicate a rather counterintuitive concept that starch accumulation in nodules is in competition with the delivery of C to the bacteroids for SNF rather than acting as a source of C. However, different species have adopted different strategies to maintain a supply of C to nodules especially during darkness (Gordon 1992), and so this may not be the case for all legumes.

3.2.2 Invertase

INV are a large family of proteins, divided by their pH optima. The acid forms are present in the cell wall or vacuole whereas the others are cytosolic with a pH optimum that is neutral or alkaline (N/A). Early findings in soybean showed that both N/A INV and SUS were present in nodules (Morell and Copeland 1984, 1985) although it was not clear which catalysed the hydrolysis of sucrose. Soluble acid INV were not detected in legume nodules initially (Gordon et al. 1999), but low activity was subsequently found in those of *L. japonicus* (Flemetakis et al. 2006). Despite there being much information on the acidic forms, there was a paucity of information about the N/A isoforms until recently. In this respect, the breakthrough was made through research on legumes.

Genomic studies in rice, Arabidopsis and poplar revealed families of genes encoding the different isoforms (Ji et al. 2005; Qi et al. 2007; Bocock et al. 2008). The gene sequences encoding the different classes of INV, however, are so different that they cannot be combined into a single phylogenetic tree. Map-based cloning of a gene in rice (*Oscyt-inv1*) and Arabidopsis (*Atcytinv1*) gave the first indication that N/A INV could have a specific effect on plant growth and development, but due to redundancy of genes in these species (Qi et al. 2007; Jia et al. 2008), the phenotype was unclear and the precise role was not found until studies were initiated on *L. japonicus*. Flemetakis et al. (2006) identified two genes expressed in *L. japonicus* roots encoding N/A isoforms that accounted for most of the activity present; only one, LjINV1, was expressed in the nodule. Subsequently, seven genes encoding N/A INV were described in *L. japonicus* (Welham et al. 2009), *LjINV1* being the most highly expressed in all organs examined. Because the main isoform, LjINV1, was NE, Flemetakis et al. (2006) proposed that it had a role in supplying hexose phosphates for nodule metabolism and SNF. Subsequently, the characterization of TILLING mutants indicated that LjINV1 was not essential for nodule formation or function but rather had a general role in growth and cell development of the whole plant (Welham et al. 2009).

Hence, SUS activity is sufficient to supply C for SNF. Since more ATP molecules are generated for each sucrose molecule converted to malate via the SUS reaction, this reaction may be favoured over the N/A INV route, the products being less dependent on ATP for further metabolism (Gordon 1992). The SUS route also supports the hypothesis that regulation of nodule activity could be achieved via a regulation of SUS activity (Gordon et al. 1997; Galvez et al. 2005).

From the data on SUS and INV alone, it is clear that NE expression can be very misleading although it is used as a rule of thumb for involving a specific gene product in nodulation, SNF and assimilation. However, if one considers that the nodule is also an organ with its own meristem, either initially as in determinate nodules (such as Lotus and soybean) or persistently as a distinct zone within indeterminate nodules (such as pea and Medicago), it is hardly surprising that genes involved with meristem development and function would not also be highly expressed when compared to whole roots, stems or leaves. Thus the true measure of nodule enhancement may be best made by comparison with meristems such as the shoot or root apex, a comparison that has never been carried out in transcript studies involving nodulation.

3.3 PEP to Malate and Transport into the Bacteroid

3.3.1 PEP Carboxylase

Phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) is a widespread enzyme present in multiple isoforms in bacteria, cyanobacteria, green algae and higher plants. In photosynthetic higher plants, different PEPC isoforms fulfil distinct physiological functions. During C_4 photosynthesis and Crassulacean acid metabolism (CAM), PEPC is responsible for the initial fixation of inorganic C in the form of bicarbonate (HCO_3^-; Izui et al. 2004). In C_3 leaves and non-photosynthetic sink organs, including seeds, roots and nodules, specific PEPC isoforms represent key players during dark CO_2 fixation serving various biochemical and physiological functions (Vidal and Chollet 1997). PEPC accounts for about 0.5–2.0 % of the soluble protein in *M. sativa* (alfalfa) and soybean nodules (Vance et al. 1994). The significance of dark CO_2 fixation during SNF will be discussed later (see Sect. 3.4). In vascular plants, PEPC is typically regulated post-translationally by metabolite allosteric effectors, including the activators glucose-6-phosphate and triose phosphates, and the inhibitors malate and aspartate (Zhang and Chollet 1997). Furthermore, PEPC activity is regulated by the reversible phosphorylation by a specific Ca^{2+}-insensitive Ser/Thr kinase, phospho*enol*pyruvate carboxylate kinase (PEPCK), also including NE isoforms (Nimmo et al. 2001; Xu et al. 2007). In *L. japonicus*, antisense-induced suppression of the NE *Ljpepc1* transcript resulted in plants showing typical nitrogen-deficiency symptoms and reduced nitrogenase activity. Furthermore, the decrease of nodule PEPC activity resulted in significant changes in SUS and asparagine aminotransferase activities coupled to lower contents for sucrose, succinate, asparagine, aspartate and glutamate (Nakagawa et al. 2003; Nomura et al. 2006) implicating PEPC in the regulation of C/N metabolic fluxes in *L. japonicus* nodules. Interestingly, the observed low efficiency of SNF in *M. truncatula*, when compared to that of alfalfa, was correlated to a significantly lower PEPC activity and organic acid content in this model legume (Sulieman and Schulze 2010).

3.3.2 Carbonic Anhydrase

One of the final products of sucrose metabolism is CO_2, which accumulates to a high level in nodules. Carbonic anhydrase (CA; carbonate dehydratase, EC 4.2.1.1) is a zinc-containing enzyme that catalyses the reversible hydration of carbon dioxide to form bicarbonate, which is the required substrate for PEP carboxylase. Several CA families (α-, β-, γ-, δ- and ζ- CAs) are present in plants, animals and microorganisms, suggesting that this simple conversion of a membrane-permeable gas into a membrane-impermeable ionic product is vital to many important biological functions. Currently, only β-CAs have been extensively studied in higher plants. In contrast, plant α-CA isoforms are biochemically and physiologically poorly characterised, including dioscorin-like nectarine (Nectarine III, NEC3) from tobacco, reported to possess both monodehydroascorbate reductase and CA activity (Carter and Thornburg 2004), and storage proteins (dioscorins, DB2 and DB3) from the yam tuber *Dioscorea batatas* which lacks CA activity (Gaidamashvili et al. 2004). Recently, γ-CA isoforms have been localised to the mitochondrial complex I of *Arabidopsis thaliana* (Sunderhaus et al. 2006).

The presence of high CA activity in nodules was reported quite early during the study of SNF (Atkins 1974). Nodule CA activity was later measured in a number of crop legumes including pea, bean and *Lupinus angustifolius* (Lane et al. 2005). The first nodule-specific CA transcripts belonging to the β-class CAs were identified in the nodules of soybean (Kavroulakis et al. 2000), alfalfa (de la Pena et al. 1997) and *L. japonicus* (Flemetakis et al. 2003), but the exact biochemical and physiological role of this CA isoform during symbiosis remains unclear. Based on transcript accumulation and localization patterns, it has been proposed that β-CAs may fulfil distinct physiological roles during nodule development. In *L. japonicus* and soybean at early stages of nodule development, the expression of *CA1* precedes the expression of nitrogenase. In addition, both *Lj*CA1 protein and CA activity are present in all cell types, co-localising with PEPC. Thus the expression of *LjCA1* has been correlated with the need to convert most of the CO_2 produced by respiration to bicarbonate, which in turn can be channelled to various biosynthetic processes including production of oxaloacetate for amino acid biosynthesis, lipogenesis, pyrimidine biosynthesis and gluconeogenesis (Kavroulakis et al. 2003; Hoang and Chapman 2002; Flemetakis et al. 2003). At later stages of nodule development, all nodule β-CAs studied so far have been found to localise to a few layers of inner cortical cells in both determinate and indeterminate nodules (de la Pena et al. 1997; Kavroulakis et al. 2000; Flemetakis et al. 2003). These cell layers appear more compact with less obvious intercellular gas spaces, compared to the immediately adjacent outer cortical cells and to the cells forming the boundary layer, and they are proposed to participate in the formation of a physical barrier to the diffusion of gases (Parsons and Day 1990). The strictly regulated spatial accumulation of β-CA transcripts has been implicated in an osmoregulatory mechanism involving the localised production of malate through the combined activities of CA and PEPC

and is analogous to the process that occurs in stomatal guard cells (de la Pena et al. 1997). Alternatively, the localization of β-CA transcripts and protein in the nodule inner cells has been proposed to facilitate the diffusion of the excess CO_2 towards the rhizosphere, in a mechanism similar to that proposed for the facilitated diffusion of atmospheric CO_2 towards the chloroplasts in photosynthetic tissues (Badger and Price 1994).

However, in all the aforementioned studies, the strictly localised expression of the nodule-specific β-CAs could not explain the high levels of CA activity detected in both the infected and uninfected cells of the central tissue in mature nitrogen-fixing nodules (Atkins et al. 2001; Flemetakis et al. 2003). This raised the possibility that alternative CA isoforms, of either plant or microbial origin and differing enough at the nucleotide level compared to β-CAs, are responsible for the observed CA activity. Recently, the availability of genome and EST sequence databases from the model legumes and their microsymbionts revealed the presence of several CA-like sequences, belonging to α- and γ- classes. In *L. japonicus*, two genes encoding NE α-CA isoforms have been indentified (Tsikou et al. 2011). Both CO_2 hydration and bicarbonate dehydration activities of the full-length proteins were demonstrated by heterologous expression. Temporal and spatial expression analysis of *LjCAA1* and *LjCAA2* revealed that both genes are induced early during nodule development and remain active in nodule inner cortical cells, vascular bundles and the central tissue during all stages of nodule development. Interestingly, both genes were slightly to moderately downregulated in ineffective nodules formed by mutant *Mesorhizobium loti* strains, indicating that these genes may also be involved in biochemical and physiological processes not directly linked to SNF and assimilation. In addition, it was recently demonstrated that also *M. loti* harbours an active periplasmic α-CA on the symbiosis island (Kalloniati et al. 2009). The *MlCaa1* gene was found to be expressed in both nitrogen-fixing bacteroids and free-living bacteria. Interestingly, gene expression in batch cultures was induced by increasing the pH of the medium. Nodulation of *L. japonicus* with a *MlCaa1* deletion mutant strain showed no differences in shoot traits and nutritional status, but the plants consistently formed more nodules and exhibited a higher fresh weight, N content, nitrogenase activity and ^{13}C content. It was proposed that although the deletion mutant does not abolish the ability to form nitrogen-fixing nodules, this α-CA may participate in an auxiliary ATP-independent mechanism that could buffer the bacteroid periplasm by providing an alternative source of protons, thus creating an environment favourable for NH_3 protonation and facilitating its diffusion and transport to the plant (Day et al. 2001; White et al. 2007). An interesting analogous mechanism to the proposed role of *MlCaa1* during symbiosis comes from the physiological role of the *Helicobacter pylori* periplasmic α-CA homologue (Fig. 3.2).

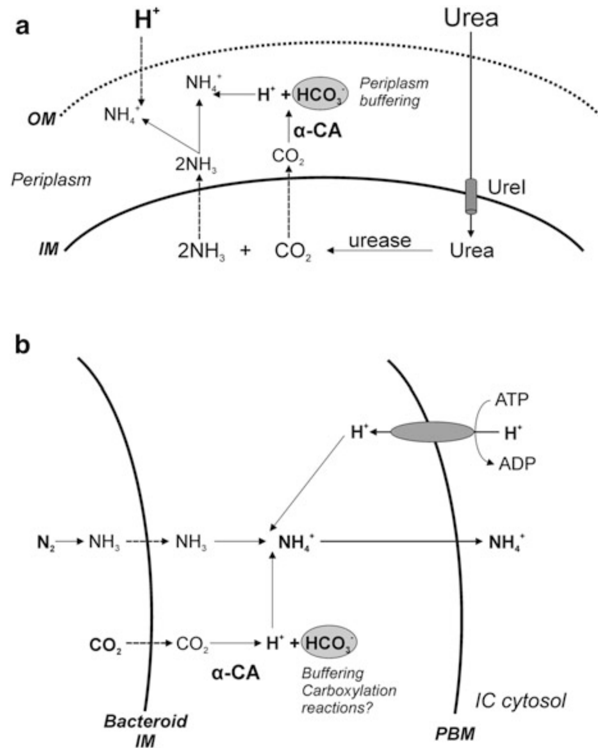

Fig. 3.2 (**a**) The role of urease and α-CA in acid acclimation of *Helicobacter pylori* (Marcus et al. 2005). *IM* inner membrane, *OM* outer membrane. (**b**) Schematic representation of the proposed role of *Ml*Caa1 during SNF. The CA catalysed hydration of the CO_2 produced by bacteroid respiration provides protons which can be used for the formation of NH_4^+, thus facilitating the diffusion of further NH_3, while the produced bicarbonate can be used for the buffering of the bacteroid periplasm. *IM* inner membrane, *PBS* peribacteroid membrane, *IC* infected cell

3.3.3 Malate Dehydrogenase

There is substantial evidence that the dicarboxylic acid (DCA) imported into the nodule is malate (reviewed by Udvardi and Day 1997; White et al. 2007). Although rhizobia do take up and can grow on sucrose and other sugars, bacteroids cannot use them, preferring malate or succinate to be most effective but also utilising formate and oxaloacetate (Herrada et al. 1989; Ou Yang et al. 1990). A plant DCA transporter has been identified in *Alnus glutinosa* that is expressed in nodules at the interface between plant cells and bacteria (Jeong et al. 2004), but in the absence of studies on specific plant mutants for DCA transporters, the exact specificity remains unknown. Furthermore, bacteroid membranes contain DCA transporters, and defects in bacteria transporters lead to ineffective nodules (Ronson et al. 1981; Udvardi and Day 1997). Malate is a key player in plant metabolism; it is a product of starch breakdown in plastids; it is utilised by mitochondria, glyoxisomes and peroxisomes; and it is the predominant DCA present in nodules (Rosendahl et al. 1990). Its level is also sensitive to SNF activity and decreases markedly in ineffective nodules (Schulze et al. 2002). There are numerous isoforms of malate dehydrogenase (MDH) in the cell, which makes analysis of enzyme activities difficult. Miller et al. (1998) characterised several isoforms in alfalfa and detected

a unique protein in the nodules whose expression was enhanced during nodulation. The gene encoding this isoform has no introns, unlike all other isoforms. This group also detected a similar protein in pea (Fedorova et al. 1999). The enzyme's K_m favoured very markedly the formation of malate from oxaloacetate. The authors therefore regarded this enzyme as a key player in the manipulation of SNF, and preliminary investigations of transgenic lines showed that some had an increase in transcript and protein levels of the enzyme and improved SNF, although this has not been followed up (Schulze et al. 2002).

In the absence of specific mutants for NE MDH, however, the essential nature of malate for SNF remains unproven. In our preliminary studies with a TILLING mutant of Lj*NEMDH* predicted to have a deleterious effect on the activity of this isoform, there was a whole plant and root cell phenotype, but nodule function was unaffected (Welham, Edwards, and Wang, unpublished). This situation is similar to that observed for *LjINV1* (see Sect. 3.2.2) where there was no effect on SNF but a profound effect on plant development (Welham et al. 2009). In alfalfa, the NE MDH isoform represents 50 % of the total activity for this enzyme in the nodule (Miller et al. 1998), but in Lotus, when using the same antibodies for immunoprecipitation (Miller et al. 1998), this isoform only appears to represent ca. 20 % of the activity (Welham, Edwards and Wang, unpublished). Hence, as for SUS activity, other MDH isoforms may also be important for nodule function, at least in *L. japonicus*.

3.4 Dark CO_2 Fixation in Nitrogen-Fixing Nodules

Several studies have demonstrated that N_2-fixing nodules are capable of fixing CO_2, while the oxaloacetate produced can be utilised either for the synthesis of malate or as a source of C skeletons for the assimilation of the symbiotically reduced N (Chollet et al. 1996). Studies using $^{14}CO_2$ have demonstrated that dark CO_2 fixation in nodules supplies the bacteroids with C skeletons, mainly in the form of organic acids, for respiration and other biochemical processes (Rosendahl et al. 1990; Vance and Heichel 1991). In addition, organic acids containing dark-fixed CO_2 can be transported from nodules to shoots of legumes (Minchin and Pate 1973; Maxwell et al. 1984; Vance et al. 1985). Recently, $Na_2{}^{13}CO_3$ labelling of *L. japonicus* roots showed that N_2-fixing nodules and both nodulated and non-nodulated roots could incorporate $^{13}CO_2$. However, nodulated plants exported significantly higher amounts of ^{13}C from roots to the shoots (Fotelli et al. 2011). In addition, dark CO_2 fixation in nodules was found to be directly linked with SNF as ineffective nodules formed by *ΔnifA* and *ΔnifH* mutant *M. loti* strains incorporated significantly less label when compared to the N_2-fixing nodules. Fix^- plants also exhibited a significant decrease in ^{13}C accumulation in leaves, an observation possibly reflecting the lack of amide amino acid export from the fix^- nodules. In contrast, ^{13}C labelling in the stems of fix^- plants remained constant, indicating that compounds other than amides may account for the ^{13}C label in stems, with organic

acids being the most prominent candidates (Fotelli et al. 2011), since significant amounts of these compounds are present in the xylem sap of amide-transporting legumes (Vance et al. 1985). Gene expression studies also revealed that transcripts for both NAD- and NADP-malic enzymes are significantly upregulated in the stems of rhizobium-inoculated plants when compared to the stems of non-inoculated plants (Fotelli et al. 2011). Interestingly, transcript accumulation in stems was only dependent on the presence of nodules rather than on SNF and export, as a similar effect was observed in plants harbouring ineffective nodules. These findings suggest that dark CO_2 fixation may fulfil multiple biochemical and physiological roles during SNF. It has been well established that CO_2 concentration in the rhizosphere can influence SNF, and prolonged elevated CO_2 supply to the root/ nodule system of alfalfa led to a significant increase in nodule CO_2 fixation, SNF and growth (Grobbelaar et al. 1971; Yamakawa et al. 2004; Fischinger et al. 2010). These beneficial effects of nodule dark CO_2 fixation are traditionally attributed to the production of malate, the principal source of energy and C skeletons for the bacteroids (Rosendahl et al. 1990; Vance and Heichel 1991). However, previous studies have demonstrated that organic acids translocated from roots to the shoots can be decarboxylated with the released CO_2 recycled through photosynthesis to form carbohydrates (Cramer and Richards 1999; Hibberd and Quick 2002). The observation that nodulation induces the expression of genes encoding malate decarboxylating enzymes in the stems of *L. japonicus* points to the possible refixation of CO_2 respired or taken up by nodules in the stems through a C_4-type mechanism. The existence of this mechanism could have a positive impact on the C budget of the plant as a whole, by at least partially reducing the C costs of nodules while at the same time maintaining a constant pool of malate in nodules, as organic acid accumulation is suggested to have a negative regulatory impact on nitrogenase activity (Le Roux et al. 2008). Future work should aim towards the quantification of the CO_2 recycled through such a mechanism and this relative contribution to the whole plant C budget.

3.5 Conclusions and Perspectives

Considerable progress has been made over the last 10 years towards our understanding of the mechanisms operating to set up a nodule, so much so that it is now timely to attempt to transfer the ability to nodulate to non-legumes (http://www.gatesfoundation.org/How-We-Work/Quick-Links/Grants-Database/Grants/2012/06/OPP1028264). Creating a nodule to receive bacteria is only part of the story, however, since many metabolic processes require adjustments before the nodule can function correctly (Udvardi and Poole 2013). The use of model legumes has helped us to determine, for example, which is the key enzyme for delivering carbohydrate to the nodule, but we still have little understanding of its regulation or which plant processes are key to nodule function since the lesions in only a few non-fixing mutants have been determined (Udvardi and Poole 2013). Many of the

enzyme isoforms required for correct functioning of carbon metabolism exist in non-legumes, in common with some of the components of signalling (Antolín-Llovera et al. 2012; Zhu et al. 2006), but their regulation will be different. As mentioned, legumes pay a significant price, which can be viewed as a 'carbon tax' on the plant, for receiving fixed nitrogen from bacteroids. This requires modifications to their metabolism and to specific isoforms involved in the process. Until we fully understand both the steps in the pathway of carbon to the nodule and its regulation, our ability to levy a tax on non-legumes or even manipulate the level of tax in legumes will be severely limited. In this regard, future attention needs to be refocussed on nodule fixation and especially those plant genes concerned with taxation.

References

Antolín-Llovera M, Ried MK, Binder A, Parniske M (2012) Receptor kinase signaling pathways in plant-microbe interactions. Annu Rev Phytopathol 50:451–473

Atkins CA (1974) Occurrence and some properties of carbonic anhydrase from legume root nodules. Phytochemistry 13:93–98

Atkins C, Smith P, Mann A, Thumfort P (2001) Localization of carbonic anhydrase in legume nodules. Plant Cell Environ 24:317–326

Badger MR, Price GD (1994) The role of carbonic-anhydrase in photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 45:369–392

Baier MC, Barsch A, Kuster H, Hohnjec N (2007) Antisense repression of the *Medicago truncatula* nodule-enhanced sucrose synthase leads to a handicapped nitrogen fixation mirrored by specific alterations in the symbiotic transcriptome and metabolome. Plant Physiol 145:1600–1618

Barratt DHP, Barber L, Kruger NJ, Smith AM, Wang TL, Martin C (2001) Multiple, distinct isoforms of sucrose synthase in pea. Plant Physiol 127:655–664

Barratt DHP, Derbyshire P, Findlay K, Pike M, Wellner N, Lunn J, Feil R, Simpson C, Maule A, Smith AM (2009) Sucrose catabolism in Arabidopsis requires cytosolic invertase but not sucrose synthase. Proc Natl Acad Sci USA 106:13124–13129

Bieniawska Z, Barratt DHP, Garlick AP, Thole V, Kruger NJ, Martin C, Zrenner R, Smith AM (2007) Analysis of the sucrose synthase gene family in Arabidopsis. Plant J 49:810–828

Bocock PN, Morse AM, Dervinis C, Davis JM (2008) Evolution and diversity of invertase genes in *Populus trichocarpa*. Planta 227:565–576

Carter CJ, Thornburg RW (2004) Tobacco Nectarin III is a bifunctional enzyme with monodehydroascorbate reductase and carbonic anhydrase activities. Plant Mol Biol 54:415–425

Chollet R, Vidal J, O'Leary MH (1996) Phospho*enol*pyruvate carboxylase, a ubiquitous highly regulated enzyme in plants. Annu Rev Plant Physiol Plant Mol Biol 47:273–298

Craig J, Barratt P, Tatge H, Dejardin A, Handley L, Gardner CD, Barber L, Wang T, Hedley C, Martin C, Smith AM (1999) Mutations at the *rug4* locus alter the carbon and nitrogen metabolism of pea plants through an effect on sucrose synthase. Plant J 17:353–362

Cramer MD, Richards MB (1999) The effect of rhizosphere dissolved inorganic carbon on gas exchange characteristics and growth rates of tomato seedlings. J Exp Bot 50:79–87

Day DA, Poole PA, Tyerman SD, Rosendahl L (2001) Ammonia and amino acid transport across symbiotic membranes in nitrogen-fixing legume nodules. Cell Mol Life Sci 58:61–71

de la Pena TC, Frugier F, McKhann HI, Bauer P, Brown S, Kondorosi A, Crespi M (1997) A carbonic anhydrase gene is induced in the nodule primordium and its cell-specific expression is controlled by the presence of Rhizobium during development. Plant J 11:407–420

Fedorova M, Tikhonovich IA, Vance CP (1999) Expression of C-assimilating enzymes in pea (*Pisum sativum* L.) root nodules: in situ localization in effective nodules. Plant Cell Environ 22:1249–1262

Fischinger SA, Hristozkova M, Mainassara Z-A, Schulze J (2010) Elevated CO_2 concentration around alfalfa nodules increases N_2 fixation. J Exp Bot 61:121–130

Flemetakis E, Dimou M, Cotzur D, Aivalakis G, Efrose RC, Kenoutis C, Udvardi M, Katinakis P (2003) A *Lotus japonicus* β-type carbonic anhydrase gene expression pattern suggests distinct physiological roles during nodule development. Biochim Biophys Acta 1628:186–194

Flemetakis E, Efrose RC, Ott T, Stedel C, Aivalakis G, Udvardi MK, Katinakis P (2006) Spatial and temporal organisation of sucrose metabolism in *Lotus japonicus* nitrogen-fixing nodules suggests a role for the elusive alkaline/neutral invertase. Plant Mol Biol 62:53–69

Fotelli MN, Tsikou D, Kolliopoulou A, Aivalakis G, Katinakis P, Udvardi MK, Rennenberg H, Flemetakis E (2011) Nodulation enhances dark CO_2 fixation and recycling in the model legume *Lotus japonicus*. J Exp Bot 62:2959–2971

Gaidamashvili M, Yuki O, Iijima S, Takayama T, Ogawa T, Muramoto K (2004) Characterization of the yam tuber storage proteins from *Dioscorea batatas* exhibiting unique lectin activities. J Biol Chem 279:26028–26035

Galvez L, Gonzalez EM, Arrese-Igor C (2005) Evidence for carbon flux shortage and strong carbon/nitrogen interactions in pea nodules at early stages of water stress. J Exp Bot 56:2551–2561

Gordon AJ (1992) Carbon metabolism in the legume nodule. In: Pollock CJ, Farrar JF, Gordon AJ (eds) Carbon partitioning within and between organisms. Bios Scientific, Oxford

Gordon AJ, Mitchell DF, Ryle GJA, Powell CE (1987) Diurnal production and utilization of photosynthate in nodulated white clover. J Exp Bot 38:84–98

Gordon AJ, Thomas BJ, Reynolds PHS (1992) Localization of sucrose synthase in soybean root nodules. New Phytol 122:35–44

Gordon AJ, Thomas BJ, James CL (1995) The location of sucrose synthase in root nodules of white clover. New Phytol 130:523–530

Gordon AJ, Minchin FR, Skøt L, James CL (1997) Stress-induced declines in soybean N_2 fixation are related to nodule sucrose synthase activity. Plant Physiol 114:937–946

Gordon AJ, Minchin FR, James CL, Komina O (1999) Sucrose synthase in legume nodules is essential for nitrogen fixation. Plant Physiol 120:867–878

Grobbelaar N, Hough MC, Clarke B (1971) Nodulation and nitrogen fixation of isolated roots of *Phaseolus vulgaris* L. 3. Effect of carbon dioxide and ethylene. Plant Soil 35:215–278

Herrada G, Puppo A, Rigaud J (1989) Uptake of metabolites by bacteriod containing vesicles and by free bacteroids from French bean nodules. J Gen Microbiol 135:3165–3177

Hibberd JM, Quick PW (2002) Characteristics of C_4 photosynthesis in stems and petioles of C_3 flowering plants. Nature 415:451–454

Hoang CV, Chapman KD (2002) Biochemical and molecular inhibition of plastidial carbonic anhydrase reduces the incorporation of acetate into lipids in cotton embryos and tobacco cell suspensions and leaves. Plant Physiol 128:1417–1427

Hohnjec N, Becker JD, Puhler A, Perlick AM, Kuster H (1999) Genomic organization and expression properties of the *MtSucS1* gene, which encodes a nodule-enhanced sucrose synthase in the model legume *Medicago truncatula*. Mol Gen Genet 261:514–522

Horst I, Welham T, Kelly S, Kaneko T, Sato S, Tabata S, Parniske M, Wang TL (2007) TILLING mutants *of Lotus japonicus* reveal that nitrogen assimilation and fixation can occur in the absence of nodule-enhanced sucrose synthase. Plant Physiol 144:806–820

Izui K, Matsumura H, Furumoto T, Kai Y (2004) Phospho*enol*pyruvate carboxylase: a new era of structural biology. Annu Rev Plant Biol 55:69–84

Jeong J, Suh S, Guan C, Tsay Y-F, Moran N, Oh CJ, An CS, Demchenko KN, Pawlowski K, Lee Y (2004) A nodule-specific dicarboxylate transporter from alder is a member of the peptide transporter family1. Plant Physiol 134:969–978

Ji XM, van den Ende W, van Laere A, Cheng SH (2005) Structure, evolution, and expression of the two invertase gene families of rice. J Mol Evol 60:615–634

Jia LQ, Zhang BT, Mao CZ, Li JH, Wu YR, Wu P, Wu ZC (2008) OsCYT-INV1 for alkaline/neutral invertase is involved in root cell development and reproductivity in rice (*Oryza sativa* L.). Planta 228:51–59

Kalloniati C, Tsikou D, Lampiri V, Fotelli MN, Rennenberg H, Chatzipavlidis I, Fasseas C, Katinakis P, Flemetakis E (2009) Characterization of a *Mesorhizobium loti* α-type carbonic anhydrase and its role in symbiotic nitrogen fixation. J Bacteriol 191:2593–2600

Kavroulakis N, Flemetakis E, Aivalakis G, Katinakis P (2000) Carbon metabolism in developing soybean nodules: the role of carbonic anhydrase. Mol Plant Microbe Interact 13:14–22

Kavroulakis N, Flemetakis E, Aivalakis G, Dahiya P, Brewin NJ, Fasseas K, Hatzopoulos P, Katinakis P (2003) Tissue distribution and subcellular localization of carbonic anhydrase in mature soybean root nodules indicates a role in CO_2 diffusion. Plant Physiol Biochem 41:479–484

Lane TW, Saito MA, George GN, Pickeing IJ, Prince RC, Morel FM (2005) Biochemistry: a cadmium enzyme from a marine diatom. Nature 435:42

Layzell DB, Pate JS, Atkins CA, Canvin DT (1981) Partitioning of carbon and nitrogen and nutrition of root and shoot apex in a nodulated legume. Plant Physiol 67:30–36

Le Roux MR, Khan S, Valentine AJ (2008) Organic acid accumulation may inhibit N_2 fixation in phosphorus-stressed lupin nodules. New Phytol 177:956–962

Marcus EA, Moshfegh AP, Sachs G, Scott DR (2005) The periplasmic alpha-carbonic anhydrase activity of *Helicobacter pylori* is essential for acid acclimation. J Bacteriol 187:729–738

Maxwell CA, Vance CP, Heichel GH, Stade S (1984) CO_2 fixation in alfalfa and birdsfoot trefoil root nodules and partitioning of ^{14}C to the plant. Crop Sci 24:257–264

Miller SS, Driscoll BT, Gregerson RG, Gantt JS, Vance CP (1998) Alfalfa malate dehydrogenase (MDH): molecular cloning and characterization of five different forms reveals a unique nodule-enhanced MDH. Plant J 15:173–184

Minchin FR, Pate JS (1973) The carbon balance of a legume and the functional economy of its root nodules. J Exp Bot 24:259–270

Morell M, Copeland L (1984) Enzymes of sucrose breakdown in soybean nodules. Plant Physiol 74:1030–1034

Morell M, Copeland L (1985) Sucrose synthase of soybean nodules. Plant Physiol 78:149–154

Nakagawa T, Izumi T, Banba M, Umehara Y, Kouchi H, Izui K, Shingo H (2003) Characterization and expression analysis of genes encoding phospho*enol*pyruvate carboxylase and phospho*enol*pyruvate carboxylase kinase of *Lotus japonicus*, a model legume. Mol Plant Microbe Interact 16:281–288

Nimmo GA, Wilkins MB, Nimmo HG (2001) Partial purification and characterization of a protein inhibitor of phospho*enol*pyruvate carboxylase kinase. Planta 213:250–257

Nomura M, Thu Mai H, Fujii M, Hata S, Izui K, Tajima S (2006) Phospho*enol*pyruvate carboxylase plays a crucial role in limiting nitrogen fixation in *Lotus japonicus* nodules. Plant Cell Physiol 47:613–621

Novak K, Pcsina K, Ncbcsarova J, Skrdleta V, Lisa L, Nasincc V (1995) Symbiotic tissue degradation pattern in the ineffective nodules of three nodulation mutants of pea. Ann Bot 76:301–313

Ou Yang L, Udvardi MK, Day D (1990) Specificity and regulation of the dicarboxylate carrier on the peribacteroid membrane of soybean nodules. Planta 182:437–444

Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Microbiol 6:763–775

Parsons R, Day DA (1990) Mechanism of soybean nodule adaptation to different oxygen pressures. Plant Cell Environ 13:501–512

Pate JS, Herridge DF (1977) Partitioning and utilization of net photosynthate in a nodulated annual legume. J Exp Bot 29:401–412

Pate JS, Layzell DB, Atkins CA (1979a) Economy of carbon and nitrogen in a nodulated and non-nodulated (NO_3-grown) legume. Plant Physiol 64:1008–1083

Pate JS, Layzell DB, McNeil DL (1979b) Modeling the transport and utilization of carbon and nitrogen in a nodulated legume. Plant Physiol 63:730–737

Qi XP, Wu ZC, Li JH, Mo XR, Wu SH, Chu J, Wu P (2007) AtCYT-INV1, a neutral invertase, is involved in osmotic stress-induced inhibition on lateral root growth in Arabidopsis. Plant Mol Biol 64:575–587

Ronson CW, Lyttleton P, Robertson JG (1981) C4-dicarboxylate transport mutants of *Rhizobium trifolii* form ineffective nodules on *Trifolium repens*. Proc Natl Acad Sci USA 78:4284–4288

Rosendahl L, Vance CP, Pedersen WB (1990) Products of dark CO_2 fixation in pea root nodules support bacteroid metabolism. Plant Physiol 93:12–19

Schulze J, Tesfaye M, Litjens RHMG, Bucciarelli B, Trepp G, Miller S, Samac D, Allan D, Vance CP (2002) Malate plays a central role in plant nutrition. Plant Soil 247:133–139

Sulieman S, Schulze J (2010) The efficiency of nitrogen fixation of the model legume *Medicago truncatula* (Jemalog A17) is low compared to *Medicago sativa*. J Plant Physiol 167:683–692

Sunderhaus S, Dudkina NV, Jänsch L, Klodmann J, Heinemeyer J, Perales M, Zabaleta E, Boekema EJ, Braun HP (2006) Carbonic anhydrase subunits form a matrix-exposed domain attached to the membrane arm of mitochondrial complex I in plants. J Biol Chem 281:6482–6488

Thummler F, Verma DPS (1987) Nodulin-100 of soybean is the subunit of sucrose synthase regulated by the availability of free heme in nodules. J Biol Chem 262:14730–14736

Tsikou D, Stedel C, Kouri ED, Udvardi MK, Wang TL, Katinakis P, Labrou NE, Flemetakis E (2011) Characterization of two novel nodule-enhanced α-type carbonic anhydrases from *Lotus japonicus*. Biochim Biophys Acta 1814:496–504

Udvardi MK, Day DA (1997) Metabolite transport across symbiotic membranes of legume nodules. Annu Rev Plant Physiol Plant Mol Biol 48:493–523

Udvardi M, Poole PS (2013) Transport and metabolism in legume-rhizobia symbioses. Annu Rev Plant Biol 64:781–805

Vance CP, Heichel GH (1991) Carbon in N_2 fixation, limitation or exquisite adaptation. Annu Rev Plant Physiol Plant Mol Biol 42:373–392

Vance CP, Boylan KLM, Maxwell CA, Heichel GH, Hardman LL (1985) Transport and partitioning of CO_2 fixed by root nodules of ureide and amide producing legumes. Plant Physiol 78:774–778

Vance CP, Gergerson RG, Robinson DL, Miller SS, Gantt JS (1994) Primary assimilation of nitrogen in alfalfa nodules: molecular features of enzymes involved. Plant Sci 101:51–64

Verma DPS, Fortin MG, Stanley J, Mauro VP, Purohit S, Morrison N (1986) Nodulins and nodulin genes of *Glycine max*. Plant Mol Biol 7:51–61

Vidal J, Chollet R (1997) Regulatory phosphorylation of C4 PEP carboxylase. Trends Plant Sci 2:230–237

Vriet C, Welham T, Brachmann A, Pike J, Perry J, Parniske M, Sato S, Tabata S, Smith AM, Wang TL (2010) A suite of *Lotus japonicus* starch mutants reveals both conserved and novel features of starch metabolism. Plant Physiol 154:643–655

Wang TL, Hedley CL (1991) Seed development in peas: knowing your three ‘r’s’ (or four, or five). Seed Sci Res 1:3–14

Wang TL, Hadavizideh A, Harwood A, Welham TJ, Harwood WA, Faulks R, Hedley CL (1990) An analysis of seed development in *Pisum sativum*. XIII. The chemical induction of storage product mutants. Plant Breed 105:311–320

Wang TL, Bogracheva TY, Hedley CL (1998) Starch: as simple as A, B, C? J Exp Bot 49:481–502

Welham T, Pike P, Horst I, Flemetakis E, Katinakis P, Kaneko T, Sato S, Tabata S, Perry J, Parniske P, Wang TL (2009) A cytosolic invertase is required for normal growth and cell development in the model legume, *Lotus japonicus*. J Exp Bot 60:3353–3365

White J, Prell J, James K, Poole P (2007) Nutrient sharing between symbionts. Plant Physiol 144:604–614

Xu W, Sato SJ, Clemente TE, Chollet R (2007) The PEP-carboxylase kinase gene family in *Glycine max* (GmPpcK1-4): an in-depth molecular analysis with nodulated, non-transgenic and transgenic plants. Plant J 49:910–923

Yamakawa T, Ikeda T, Ishizuka J (2004) Effects of CO_2 concentration in rhizosphere on nodulation and N_2 fixation of soybean and cowpea. Soil Sci Plant Nutr 50:713–720

Zhang XQ, Chollet R (1997) Phospho*enol*pyruvate carboxylase protein kinase from soybean root nodules: partial purification, characterization, and up/down-regulation by photosynthate supply from the shoots. Arch Biochem Biophys 343:260–268

Zhu H, Riely BK, Burns NJ, Ané J-M (2006) Tracing nonlegume orthologs of legume genes required for nodulation and arbuscular mycorrhizal symbioses. Genetics 172:2491–2499