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New perspectives on nodule nitrogen assimilation in actinorhizal symbioses

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Abstract. Nitrogen-fixing root nodules are plant organs specialised for symbiotic transfer of nitrogen and carbon between microsymbiont and host. The organisation of nitrogen assimilation, storage and transport processes is partitioned at the subcellular and tissue levels, in distinctive patterns depending on the symbiotic partners. In this review, recent advances in understanding of actinorhizal nodule nitrogen assimilation are presented. New findings indicate that *Frankia* within nodules of *Datisca glomerata* (Presl.) Baill. carries out both primary nitrogen assimilation and biosynthesis of arginine, rather than exporting ammonium. Arginine is a typical storage form of nitrogen in plant tissues, but is a novel nitrogen carrier molecule in root nodule symbioses. Thus *Frankia* within *D. glomerata* nodules exhibits considerable metabolic independence. Furthermore, nitrogen reassimilation is likely to take place in the host in the uninfected nodule cortical cells of this root nodule symbiosis, before amino acid export to host sink tissues via the xylem. The role of an augmented pericycle in carbon and nitrogen exchange in root nodules deserves further attention in actinorhizal symbiosis, and further highlights the importance of a comprehensive, structure–function approach to understanding function in root nodules. Moreover, the multiple patterns of compartmentalisation in relation to nitrogen flux within root nodules demonstrate the diversity of possible functional interactions between host and microsymbiont that have evolved in the nitrogen-fixing clade.

Additional keywords: *Datisca glomerata*, *Frankia*, nitrogen fixation, root nodule, symbiosis.

Introduction

The biological reduction of atmospheric dinitrogen (nitrogen fixation) is the fundamental process that provides essential nitrogen to the biosphere as ammonium. A major fraction of symbiotic nitrogen fixation worldwide is contributed by actinorhizal symbioses formed by the association between soil-dwelling, Gram-positive actinobacteria of the genus *Frankia* and a group of more than 200 mostly woody plant species from eight different families, collectively called actinorhizal plants. Actinorhizal plants fall into three phylogenetically-related groups (see review by Pawlowski *et al*. 2011): Fagales (Betulaceae, Casuarinaceae, Myricaceae), Cucurbitales (Datiscaceae, Coriariaceae) and Rosales (Rosaceae, Elaeagnaceae, Rhamnaceae), which, together with the legumes (Fabales), form a single 'nitrogen-fixing clade' within the angiosperms (Soltis *et al*. 1995).

In all of these symbioses, the host plants form root nodules, highly-specialised organs for nitrogen production, wherein the bacteria carry out nitrogen fixation while being supplied by the plant with photosynthetically-derived carbon. Understanding the functional partitioning of carbon and nitrogen fluxes in root nodules is of key importance in unravelling the molecular and evolutionary basis of symbiotic adaptation between the partners. This partitioning is very complex, involving subcellular metabolic interactions between the host organelles and the microsymbiont, and higher-order patterns of cell–cell interaction and tissue specialisation, to enable metabolite transport and the sequential steps of metabolite transformation (Schubert 1986; Lodwig *et al*. 2003; Valverde and Huss-Danell 2008).

For legume symbioses and many of the actinorhizal symbioses, strategies for functional partitioning of nodule

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nitrogen assimilation have been characterised, and integrative models have been presented (Schubert 1986; Lodwig *et al*. 2003; Prell and Poole 2006; White *et al*. 2007; Valverde and Huss-Danell 2008). In the majority of legume and actinorhizal symbioses, the microsymbionts provide the plant with the products of nitrogen fixation, primarily in the form of ammonium, as inferred using isotopically labelled N_2 (Bond *et al*. 1958; Baker and Parsons 1997; Scharff *et al*. 2003). Although nitrogen fixation takes place in the microsymbiont (*Frankia* or rhizobia), it is not clear from the isotope studies whether primary ammonium assimilation occurring via the glutamine synthetase–glutamate synthase (GS–GOGAT) cycle is performed by the bacterium or by the plant host.

Nodole primary nitrogen assimilation

Molecular analyses have shown that plant cytosolic glutamine synthetase (GS) was expressed at high levels in the infected cells of legume root nodules (alfalfa (*Medicago sativa* L.; Temple *et al*. 1995), *Phaseolus vulgaris* L. (Forde *et al*. 1989) and soybean (*Glycine max* L.; Miao *et al*. 1991)). The question of GS localisation has been answered for several actinorhizal taxa by a combination of enzyme activity studies, localisation of cytosolic GS protein (Hirel *et al*. 1982) and cytosolic GS transcripts (Guan *et al*. 1996) in nodules of *Alnus glutinosa* L. (Betulaceae); and by immunodetection in *Discaria trinervis* (Hook et Arn.) (Rhamnaceae; Valverde and Wall 2003). In *A. glutinosa*, the cytoplasmic GS is localised in the *Frankia*-infected cells (Hirel *et al*. 1982; Guan *et al*. 1996) and in the pericycle of the nodule vascular system (Guan *et al*. 1996). A reciprocal repression of *Frankia* GS in symbiosis, relative to the free-living, nitrogen-fixing condition, has been shown to occur in *A. glutinosa* at the protein (Lundquist and Huss-Danell 1992) and transcript (Alloisio *et al*. 2010) levels. Taken together, the evidence indicates that in this system, the immediate product of nitrogen fixation exported from *Frankia* to the surrounding host cytoplasm must predominantly be NH_3 or NH_4^+. In the host cytosol, ammonium is then assimilated via the GS–GOGAT pathway. Given that the perisymbiont space is acidic in legume symbioses (Kannenberg and Brewin 1989) and in arbuscular mycorrhizal symbioses (Guttenberger 2000), it is likely that in this system, the fixed nitrogen diffuses through the *Frankia* membrane in the form of NH_3, is protonated in the perisymbiont space to NH_4^+ and is actively transported across the perisymbiont membrane into the cytosol.

For the host plant, root nodules represent nitrogen sources. Nitrogen assimilated as glutamate and glutamine via the GS–GOGAT pathway is further assimilated to amino acids that can serve as nitrogen carriers or temporary storage forms (i.e. amino acids with a relatively high N : C ratio). Nitrogen in the form of amino acids is then transported to the aboveground nitrogen sinks via the xylem as 'export' amino acids. In several actinorhizal symbioses, more so than in legumes, there appears to be an intervening nitrogen reassimilation step before export from the nodule, since the composition of export amino acids is not necessarily what predominates in the nodule (see discussion below and table 1 in Valverde and Huss-Danell 2008). It is thus useful to separate these two functions – synthesis of carrier amino acids that can serve as temporary storage forms within the nodule, and subsequent formation of transport forms of nitrogen – in considering nitrogen assimilation and flux in actinorhizal nodules. As we are beginning to learn, these steps can involve distinct and specialised partitioning of assimilatory and reassimilation pathways.

An analysis of amino acids accumulated in different actinorhizal symbioses shows some patterns, shown in Table 1. Fixed nitrogen in the nodule can be stored as an amide (i.e. asparagine) or as urea-cycle amino acids (i.e. citrulline or arginine). Asparagine contains two nitrogen atoms per molecule, citrulline has three and arginine has four nitrogen atoms per molecule. Among the actinorhizal symbioses, the asparagine pathway predominates in nodules of several genera (e.g. *Myrica*, *Hippophae*, *Elaeagnus*, *Ceanothus* and *Casuarina*); the arginine pathway, including citrulline, is particularly activated in nodules of the genera *Alnus*, *Coriaria* and *Datisca*. This is depicted in Table 1, where the principal nodule amino acids for most taxa examined are glutamine, glutamate, and either asparagine, citrulline or arginine. In the

Table 1. Principal amino acids in nodules of actinorhizal species, adapted from Wheeler and Bond (1970) and Berry *et al*. (2004), expressed as % total amino acids

NS, not separated, or absent or present at low levels, and hence included with other amino acids; Cit, citrulline; Arg, arginine; Asn, asparagine; Glu, glutamic acid; Asp, asparatic acid

Actinorhizal species	% of total amino acids						
	Cit	Arg	Asn	Glu	Gln	Asp	Other
Alnus glutinosa	39.3	NS	NS	24.5	9.4	7.9	18.9
Alnus inokumai	19.3	NS	21.5	20.9	22.2	1.8	14.3
Myrica gale	0	NS	53.6	11.2	17.5	2	15.7
Myrica cerifera	0	NS	58.4	9.9	13.5	8.2	10
Myrica pilulifera	0	NS	38.6	23.2	2.2	22	14
Myrica cordifolia	0	5.3	69	5.9	11.9	1.6	6.3
Hippophae rhamnoides	0	NS	61.7	13.1	3.2	7.1	14.9
Elaeagnus angustifolia	0	NS	57	8.4	10.6	12	12
Ceanothus velutinus	0	6.2	83	1.8	3.3	1.8	3.9
Casuarina cunninghamiana	0	NS	74.3	2.2	17.7	3.3	2.5
Coriaria myrtifolia	0	32	4.2	22.8	23.7	4.3	13
Datisca glomerata	n/s	26	trace	17.3	33.1	4.9	18.7

case of a few species (especially *Alnus inokumai* Murai & Kusaka, but also *Myrica cordifolia* (L.) and *Ceanothus velutinus* Dougl. var. *laevigarus* Torr. and Gray), it appears that, based on patterns of amino acid content (Table 1), both carrier pathways may be present. Given the rapid rate of amino acid metabolism, detecting a distribution in a suite of amino acids in nodule extracts is to be expected. Amides are the predominant nodule nitrogen carriers in legume nodules, although some legumes of tropical origin produce and export ureides instead (Schubert 1986). Interestingly, although many legumes utilise asparagine as a nodule carrier compound, the arginine biosynthetic pathway is apparently not utilised for nitrogen storage or export in legume nodules (Schubert 1986). It would be of interest to examine the principal nodule amino acids in the rosaceous actinorhizal symbioses (genera *Purshia*, *Cercocarpus*, *Dryas*, etc.), which have not yet been characterised.

Even though the arginine biosynthetic pathway is utilised in both *Alnus* and *Datisca* symbioses, recent findings have shown that metabolic partitioning in the nodule tissue is surprisingly different. A novel pattern of functional compartmentalisation of both the GS–GOGAT pathway and arginine biosynthesis between host and *Frankia* is operating in the *Datisca* nodule. Earlier, we showed that host GS was not detectable, either at the transcript or the protein level, in the infected cells of *Datisca glomerata* (Presl.) Baill. nodule (Berry *et al*. 2004). Instead, high levels of plant cytosolic GS expression were demonstrated in the uninfected cortical cells that surround the infected tissue. It was concluded that *Frankia* could not be exporting ammonium without induction of GS in the infected tissue, since ammonium accumulation in plant cells is toxic (Berry *et al*. 2004). Therefore, an alternate form of nitrogen must be transferred from *Frankia* to the host-infected cell. It was hypothesised that *Frankia*, not the host, must carry out primary ammonium assimilation in *D. glomerata*.

Frankia carries out both the first step in assimilation of the products of nitrogen fixation and arginine biosynthesis in nodules of *D. glomerata*

Based on sequence information from the recently published draft genome of the unculturable *Frankia* symbiont in root nodules of *D. glomerata* (NCBI GenBank accession number CP002801; 5.3 Mb, 70% G + C), semiquantitative reverse transcriptase PCR (RT-PCR) was used to detect expression levels of *Frankia* genes encoding enzymes of primary nitrogen assimilation (GSI, GSII and two glutamate oxo-glutarate aminotransferase (GOGAT) proteins) in this symbiosis (Table 2). Gene expression levels of three genes encoding enzymes from the arginine biosynthetic pathway were also tested. These enzymes represented three key segments of the arginine biosynthetic pathway: carbamoyl phosphate synthase (CPS; E.6.3.55) catalyses the conversion of glutamine to carbamoyl phosphate; ArgJ is a bifunctional enzyme (EC.2.3.1.35, E.C.2.3.1.1) that catalyses the conversion of glutamate to ornithine via the *N*-acetyl glutamate cycle. The end products of these two pathways, CP and ornithine, are combined to form citrulline, an intermediate precursor of arginine. The third gene tested for expression, argininosuccinate lyase or ArgH (E.4.3.2.1), catalyses the final step in arginine biosynthesis. Expression of *nifH*, encoding one of the subunits of the nitrogen-fixing enzyme complex nitrogenase, was used as a basis for the comparison of expression levels among these genes in RNA extracts of nodules grown in N-depleted conditions (see Supplementary methods available as an Accessory publication to this paper). As shown in Table 2, expression of a GOGAT gene (*gltS ss*) was very high relative to *nifH* expression (0.68). Glutamine synthetase I (*glnA*), CPS (*carB*), *argJ* and *argH* were all expressed at comparable levels, from 0.21 to 0.27 in relation to *nifH* expression. On the other hand, expression of the gene encoding the second bacterial glutamine synthetase type, *glnII*, was comparatively low, as were the expression levels of the genes encoding ferredoxin-dependent GOGAT (*gltS fd*) and urease (α subunit; *ureC*). This pattern contrasts sharply with a *nifH*-based comparison of *Frankia* gene expression in nodules of *A. glutinosa* using microarray-based gene expression data (Tables 3, 4; based on Alloisio *et al*. 2010). While caution must be exercised in interpreting metadata derived from two different experiments (Tables 2–4), it can be seen that for *Frankia* in symbiosis with *A. glutinosa*, *glnA* and *glnII* expression relative to *nifH* is detectable in a range from 0.06 to 0.2, whereas the expression levels of genes encoding enzymes of the arginine biosynthetic pathway genes are an order of magnitude lower. Moreover, arginine pathway enzyme genes are not among those upregulated in *A. glutinosa* nodules as compared with free-living *Frankia* (Alloisio *et al*. 2010). The latter result would be expected, since citrulline is synthesised in the host (Guan *et al*. 1996), and plant GS is expressed in infected *Alnus* cells (Hirel *et al*. 1982; Guan *et al*. 1996).

Table 2. Semiquantitative RT-PCR of selected *Frankia* genes for amino acid biosynthesis in nodules of *D. glomerata*, calculated as signal intensity ratio with respect to *nifH*

$n = 2$. *argJ*, ornithine acetyltransferase; *argH*, argininosuccinate lyase; *carB*; carbamoyl phosphate synthase large subunit; *gltS* (*ss*), NADH- or NADPH-dependent glutamate synthase, small subunit, ZP_06475529.1; *gltS* (*fd*), annotated as ferredoxin-dependent glutamate synthase, ZP_06475530.1; *glnA*, glutamine synthetase I; *glnII*, glutamine synthetase II; *ureC*, urease α-subunit. Experimental details are given in the Accessory publication to this paper

	Mean	±s.e.
nifH	1.000	–
argJ	0.212	0.160
argH	0.220	0.009
carB	0.270	0.010
gltS (ss)	0.680	0.500
gltS (*fd*)	0.010	0.002
glnA	0.230	0.090
glnII	0.070	0.040
ureC	0.030	0.010

There is precedent for some aspects of the alternative N export strategy of *Frankia* in *D. glomerata* nodules, in that alanine and other amino acids were detected as products of bacteroid nitrogen fixation and export in rhizobial symbioses with pea (*Pisum sativum* L.) and soybean, particularly in conditions of high bacteroid cell density or at high ammonium concentration (Lodwig *et al*. 2003). In conditions of high

Table 3. Gene expression microarray data for selected *Frankia* genes for amino acid biosynthesis in *A. glutinosa* nodules

Calculated from raw fluorescence values by N. Alloisio (Centre National de la Recherche Scientifique, Université de Lyon, France), from experiment 1 in Alloisio *et al*. (2010), and expressed as signal intensity ratio with respect to *nifH*. $n = 3$. *argJ*, ornithine acetyltransferase; *argH*, argininosuccinate lyase; *carB*; carbamoyl phosphate synthase large subunit; *gltS* (*ss*), NADH- or NADPH-dependent glutamate synthase, small subunit, ZP_06475529.1; *gltS* (*fd*), annotated as ferredoxin-dependent glutamate synthase, ZP_06475530.1; *glnA*, glutamine synthetase I; *glnII*, glutamine synthetase II; *ureC*, urease α-subunit

	Mean	±s.e.
nifH	1	–
argJ	0.0083	0.0003
argH	0.0058	0.0008
carB	0.0090	0.0008
gltS (*ss*)	0.0232	0.0029
gltS (*fd*)	0.0214	0.0026
glnA	0.2102	0.0493
glnII	0.0889	0.0132
ureC	0.0067	0.0006

Table 4. Gene expression microarray data for selected *Frankia* genes for amino acid biosynthesis in *A. glutinosa* nodules, calculated from raw fluorescence values by N. Alloisio (CNRS, Université de Lyon, France), from experiment 3 in Alloisio *et al*. (2010, expressed as signal intensity ratio with respect to *nifH*

$n = 3$. *argJ*, ornithine acetyltransferase; *argH*, argininosuccinate lyase; *carB*; carbamoyl phosphate synthase large subunit; *gltS* (*ss*), NADH- or NADPH-dependent glutamate synthase, small subunit, ZP_06475529.1; *gltS* (*fd*), annotated as ferredoxin-dependent glutamate synthase, ZP_06475530.1; *glnA*, glutamine synthetase I; *glnII*, glutamine synthetase II; *ureC*, urease α-subunit. Note that in the *A. glutinosa* data, difference in relative expression of *glnA* and *glnII* between Tables 3, 4 may be related to somewhat different timing of nodule harvest or other experimental differences from the independent laboratories who carried out the experiments (N. Alloisio, pers. comm.)

	Mean	±s.e.
nifH	1	–
argJ	0.0063	0.0007
argH	0.0025	0.0002
carB	0.0055	0.0003
gltS(*ss*)	0.0192	0.0019
gltS(*fd*)	0.0207	0.0009
glnA	0.0561	0.0111
glnII	0.0634	0.0053
ureC	0.0023	0.0002

ammonium accumulation, alanine dehydrogenase in the bacteroid can be activated for primary nitrogen assimilation, in addition to ammonium secretion. Nevertheless, under most experimental conditions in pea, amino acid secretion represented only one form of total nitrogen excretion by rhizobia in these symbioses, the remainder being ammonia (Lodwig *et al*. 2003).

The situation in *Datisca glomerata*-type nodules appears, therefore, to be a markedly different variation from all other root nodule symbioses, in that no ammonium is exported into the cytosol of the infected host cells. In symbiotic *Frankia* in *D. glomerata* nodules, nitrogen fixation, as signified by *nifH* expression, is confined to the radially oriented finger-shaped vesicles that form a ring around the central vacuole of the infected cells (Pawlowski *et al*. 2003). As can be seen in Fig. 1*b*, *Frankia* vesicles within the infected cortical cells of *D. glomerata* are extremely tightly-arrayed, typical for nodules of the actinorhizal Cucurbitales (*Datisca* sp. and *Coriaria* sp.; Newcomb and Pankhurst 1982; Hafeez *et al*. 1984). A blanket of mitochondria that surrounds the *Frankia* vesicles further reduces oxygen concentration within the vesicles, which, in combination with a *Frankia*-truncated globin and high internal respiration, is presumably sufficient to allow nitrogen fixation by the oxygen-sensitive nitrogenase enzyme complex (Silvester *et al*. 1999; Tjepkema *et al*. 1999; Pawlowski *et al*. 2007). The vegetative hyphae are concentrated in the peripheral cytoplasm of the infected host cells, outside the mitochondrial oxygen barrier, potentially creating two *Frankia* compartments with very different redox conditions. Portions of the N assimilatory metabolism may occur outside the mitochondrial blanket (i.e. in the *Frankia* hyphae). This possibility needs to be explored with further localisation studies.

At any rate, evidence of complex pathways of nitrogen assimilation by *Frankia* in symbiosis with *D. glomerata* supports an evolutionary scenario of a more metabolically independent model for *Frankia* symbioses, as suggested by Alloisio *et al*. (2010). In that study, based on microarray comparison between microsymbiont gene expression pattern in *A. glutinosa* nodules and nodules of the model legume *Medicago truncatula* Gaertn., it was concluded that *Frankia* retains more metabolic independence in symbiosis than rhizobia, which is possibly an evolutionarily primitive trait. If the *Frankia* symbiont of *D. glomerata* exhibits a greater degree of independent metabolism in association with the host, then *Frankia* in symbiosis with Cucurbitales might represent a basal condition among the actinorhizal symbioses, an assumption supported by some phylogenetic analyses which have placed the *Frankia* symbiont of the *Datisca*–*Coriaria* group as basal to other *Frankia* (Jeong *et al*. 1999), and the Cucurbitales as the basal clade of actinorhizal plants (Pawlowski *et al*. 2003). Since this *Frankia* symbiont also nodulates hosts in other actinorhizal families (e.g. Rosaceae, some Rhamnaceae), wider comparisons at the microsymbiont genomic level are needed to define the evolutionary framework.

The uninfected cortical tissue in *Datisca glomerata* is the site of N reassimilation

We do not yet know the identity of the nitrogen-containing compound(s) exported from *Frankia* to the host in the *Datisca*–*Coriaria* nodule type. The most abundant amino acids by far in nodule extracts are glutamine, glutamate and arginine. No detectable amounts of citrulline accumulate in the nodules (Wheeler and Bond 1970), nor was ornithine abundant (Berry *et al*. 2004). To unravel the possibilities, it is important to consider the structure–function relationships of the nodule tissues specifically in relation to synthesis of nodule nitrogen storage compounds. The high level of plant cytosolic GS (gene and protein) detected in the uninfected cortical tissue indicates

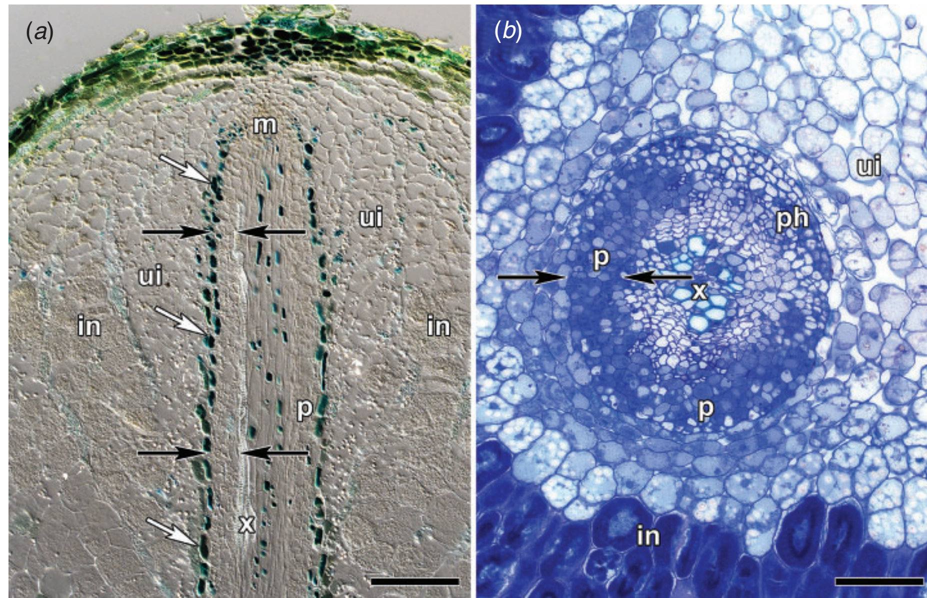

Fig. 1. (*a*) Longitudinal section of an *Alnus glutinosa* nodule lobe and (*b*) cross-section of a *Datisca glomerata* nodule lobe. **In**, infected cortical cells; **m**, meristem; **p**, pericycle; **ph**, phloem; **ui**, uninfected cortical cells (often containing starch grains); **x**, xylem. White arrows in (*a*) point to cells in the endodermis (containing flavan-filled vacuoles); black arrows in both (*a*) and (*b*) depict the extent of the pericycle. *A. glutinosa* nodules were fixed as described by Rashidi *et al*. (2011), embedded in Steedman's wax according to Vitha *et al*. (1997); sections 10 µm thick were cut on a rotary microtome (HM360; Microm, Walldorf, Germany) and stained with 0.01% toluidine blue (Sigma-Aldrich, Munich, Germany) in dd H_2O. *D. glomerata* nodules were fixed in the same way and embedded in Technovit 7100 (Heraeus-Kulzer, Wehrheim, Germany), then sectioned and stained using a mixture of 0.01% ruthenium red (Sigma-Aldrich) and 0.01% toluidine blue (Sigma-Aldrich) in a borate buffer (Rashidi *et al*. 2011). Panel (*a*) is imaged with differential interference contrast. Scale bars denote 100 µm.

that glutamine must be assimilated in this tissue from free ammonium. Moreover, while arginine is an abundant amino acid in the nodule, only glutamate and glutamine, with some aspartate, were detected in xylem exudate in *D. glomerata.* The model proposed by Berry *et al*. (2004) suggested that these findings could be accounted for by the operation of the urea cycle in the uninfected cortical tissue (e.g. arginine catabolism, release of urea and liberation of ammonium via urease; see Witte 2011). Although it remains to be confirmed experimentally, it seems likely that the *D. glomerata* nodule functions metabolically in a fashion similar to seed tissue. Many plants, including, specifically, legumes, cucurbits and several trees, synthesise arginine in plastids in the cotyledons of developing seeds and accumulate this compound as a storage form of nitrogen (Witte 2011). During seed germination, arginine is catabolised in the mitochondria via enzymes of the urea cycle, resulting in ornithine with a byproduct of urea. Urea is exported to the cytoplasm where it is broken down into ammonium by urease (Funck *et al*. 2008).

In nodules of *D. glomerata*, it would be logical to export arginine itself from *Frankia* to the host cells, with *Frankia* fulfilling a plastid-like organellar function. A transporter that exports basic amino acids has been characterised in the actinobacterial taxon, *Corynebacterium* (Eggeling and Sahm 2003). It is also possible that arginine biosynthesis and catabolism occur both in *Frankia* and the uninfected host cortex, via metabolic partitioning. In this scenario, either glutamine or glutamate could be exported from *Frankia*. However, given the low level of *ureC* expression detected in *Frankia* by RT-PCR (Table 2) and the high energetic cost of maintaining both pathways in both organisms, this is the less likely possibility. Further studies, including *in situ* localisation of gene expression, and comprehensive transcriptome analysis, are warranted to gain a more definitive understanding of these pathways and their compartmentalisation.

Continuity of transport: the nitrogen 'pipeline'

Still unresolved is the question of symplastic versus apoplastic transport of amino acids from *Frankia* in the infected cells, through the uninfected cortical tissue, and to the nodule vascular tissue. Unlike the nodule nitrogen carrier forms (asparagine, citrulline and arginine), the composition of amino acids transported out of nodules into the xylem in actinorhizal plants shows a substantial diversity, more than has been reported in legumes, as has been thoroughly reviewed by Valverde and Huss-Danell (2008). This diversity in xylem transport compounds indicates that a considerable degree of reassimilation of nitrogen within actinorhizal nodules before export must take place.

In nodules of *D. glomerata*, as has been recently shown (Schubert *et al*. 2011), there is a strong network of plasmodesmatal connections both within the infected cortical tissue, and between the infected and uninfected cortical cells. If amino acids were exported from *Frankia* to the infected

host cells, they could well be swept along via a concentration gradient within the symplastic continuum to the uninfected cells, where degradation and ammonium reassimilation would take place. Such a hypothetical pathway of nodule nitrogen flux in this symbiosis is illustrated in Fig. 2. This model assumes that amino acids are first effluxed from *Frankia* via a bacterial amino acid transporter into the perisymbiont space, then transported across the host-derived perisymbiont membrane. A plant amino acid transporter with high affinity for arginine and other basic amino acids has been characterised in *Arabidopsis thaliana* (L.) Heynh. (Frommer *et al*. 1995). An apoplastic pathway via the perisymbiont space could also be postulated, although it would be less efficient and amino acids must eventually enter the symplast of the uninfected cells by crossing a host unit membrane. Once within the uninfected cortical cells, arginine could be catabolised to ammonium and glutamate, and subsequently to glutamine for export to the vascular tissue. Whether there is then symplastic continuity in actinorhizal nodules from the cortex through the endodermis and pericycle before export to the xylem, or whether apoplastic transport operates somewhere between the cortex and the vascular tissue, remains to be investigated.

The pericycle is multilayered in some actinorhizal nodules, and plays a pivotal role in carbon and nitrogen exchange

Plant cytoplasmic *GS* gene expression is localised in two tissues in nodules of *A. glutinosa* and *A. incana*: in the mature *Frankia*-infected cortical cells and in the pericycle (Guan *et al*. 1996; K. Pawlowski and P.-O. Lundquist, unpubl. data). The infected cortical cells are also the site of expression of acetylornithine transaminase (AOTA) in *A. glutinosa*, a key enzyme in citrulline biosynthesis (Guan *et al*. 1996). AOTA, however, is not expressed in the pericycle. The presence of GS in the pericycle led Guan *et al*. (1996) to postulate that this tissue could function in the breakdown of citrulline and the reassimilation of ammonium before export into the xylem, because glutamate is enriched in the xylem compared with nodules (Blom *et al*. 1981).

The pericycle, shown in Fig. 1*a*, is a distinctive tissue in *A. glutinosa* because of its unusual multiseriate formation, first described by Burgess and Peterson (1987). Notably, the pericycle in nodules of *D. glomerata* also exhibits formation of additional cell layers (Fig. 1*b*). These cell divisions in the pericycle are particularly remarkable in the horseshoe-shaped side of the pericycle adjacent to the sector of uninfected cortical cells that directly abut the *Frankia*-infected nodule tissue. The formation of additional cell layers in the pericycle, as well as the large, often starch-filled cells seen in the uninfected cortex, contribute to the structural and functional asymmetry in the Cucurbitales nodule type (see review by Pawlowski *et al*. 2011).

The pericycle is the only tissue in developing plant roots that does not lose its meristematic potential (Dubrovsky *et al*. 2000). Lateral root primordia arise in the pericycle, as do actinorhizal nodule primordia (reviewed in Newcomb and Wood 1987). Meristematic activity in the pericycle is known to be regulated by both auxin and sucrose (Nieuwland *et al*. 2009). Sucrose

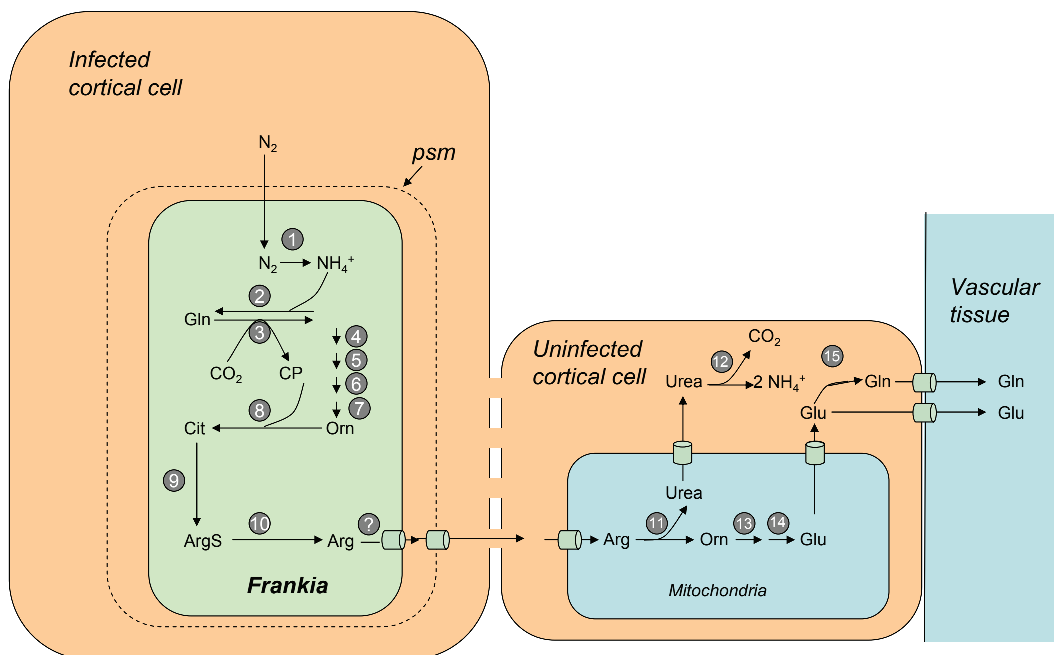

Fig. 2. A schematic representation of possible pathways of nitrogen assimilation and routes of nitrogen transport in root nodules of *Datisca glomerata*, indicated with arrows. 1, nitrogenase; 2, glutamine synthetase (GS); 3, glutamate synthase (4–glutamate oxo-glutarate aminotransferase; GOGAT); 4, *N*-acetylglutamate synthase; 5, acetylglutamate kinase; 6, *N*-acetylglutamate reductase; 7, acetylornithine aminotransferase; 8, ornithine transcarbamoylase; 9, argininosuccinate synthase; 10, argininosuccinate lyase; 11, arginase; 12, urease; 13, mitochondrial δ-ornithine aminotransferase; 14, pyrroline-5-carboxylate dehydrogenase; 15, GS–GOGAT. PSM, perisymbiont membrane; (?), export process not yet demonstrated. See 'Continuity of transport: the nitrogen 'pipeline'' section for detailed description.

synthase is expressed in the pericycle and infected tissue of *A. glutinosa* nodules at high levels (Van Ghelue *et al*. 1996) and in the infected tissue of *D. glomerata* nodules (Schubert *et al*. 2011), although not in the pericycle in the latter host. It is likely that in these actinorhizal root nodules, where sucrose transport from the phloem to the infected tissue must be high and where amino acid export from the cortex to the xylem must similarly be very active, the nodule pericycle plays an active role in mediating carbon import and nitrogen export. Nevertheless, some variability in the tissue specificity of nitrogen reassimilation is to be expected. For example, the nodule pericycle in *Casuarina glauca* Sieber does not appear to be highly multiseriate (K. Pawlowski and K. Demchenko, unpubl. data). More extensive functional and developmental studies of the pericycle in actinorhizal nodules are warranted.

Conclusion

As genome-based data become increasingly available, researchers will continue to elucidate the complex functional interactions between hosts and microsymbiont partners that have evolved in different actinorhizal symbioses. The importance of understanding structure–function relationships in root nodules, at spatial scales from the subcellular to the tissue level, cannot be overemphasised in exploring principles of commonality and divergence in the evolution of root nodule symbiosis. In relation to nitrogen flux, it is useful to distinguish between nodule nitrogen carrier molecules that can serve as temporary storage, and those amino acids that are exported from the nodule. It is perhaps not surprising that in the *Datisca* nodule type, arginine, a basic amino acid, is not observed to be exported into the acidic xylem sap. Nevertheless, in this and in other nodule symbioses, evidence indicates that reassimilation of nitrogen is an important step before export from the nodule to host plants' nitrogen sinks. The relative metabolic independence of *Frankia* within the host tissue with respect to nitrogen assimilation is another theme that is worthy of further investigation in an evolutionary context, particularly in comparing regulatory control of the microsymbiont by the host, both within the actinorhizal subclade, and between actinorhizal and legume symbioses. The diversity of ways in which the two partners in different root nodule symbioses compartmentalise similar functional activities is truly remarkable. The unique patterns of nodule structure and function observable in the Cucurbitales marks a significant outlier that can, perhaps, shed light on the evolutionary basis of root nodule symbiosis. Genomic and transcriptomic sequencing for several actinorhizal symbioses is surely the next important step, combined with techniques of spatial and temporal localisation at the molecular level.

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