

*nil*, and it was shown that *35S::PnCO* promoted flowering in the *co* mutants of *Arabidopsis* under both LD and SD conditions<sup>20</sup>. These findings suggest the existence of common mechanisms for the photoperiodic control of various processes in diverse plant species. □

Methods

Plant materials and growth conditions

Japanica rice cultivars, Norin 8, *OsGI*-ox transgenic plants and *OsGI*-RNAi transgenic plants were grown in climate chambers with 24-h temperature cycles (12 h, 30 °C during subjective day; 12 h, 25 °C during subjective night). The fluence rates of light were ~300 μmol m<sup>-2</sup> s<sup>-1</sup> (400–750 nm) under LD and SD conditions.

Generation of *OsGI*-ox and *OsGI*-RNAi transgenic plants

To generate *OsGI*-ox transgenic rice, *OsGI* cDNA (containing a 240-base-pair (bp) and a 274-bp fragment as 5' and 3' untranslated regions, respectively) was introduced into a Ti-based vector in which *OsGI* is driven by the maize *Ubi1* promoter<sup>21</sup>. To produce the hairpin RNAi construct, two 3.2-kilobase (kb) *OsGI* cDNAs were fused in reverse orientation, and a 28-bp unrelated fragment was inserted as a linker. This construct was fused with the maize *Ubi1* promoter. *Agrobacterium*-mediated transformation of rice was performed as described<sup>22</sup>. Transformed calluses were selected by hygromycin resistance, and plants were regenerated from transformed calluses.

Polymerase chain reaction with reverse transcription (RT-PCR)

*OsGI*, *Hd1* (*Se1*), *Hd3a* and *ubq* sequences were amplified by PCR with the primers listed in Supplementary Table 1. For the PCR experiments shown in Supplementary Fig. 2, conditions of 24, 28, 28 and 24 cycles were used for the amplification of *OsGI*, *Hd1* (*Se1*), *Hd3a* and *ubq*, respectively. For the PCR experiments shown in Fig. 3b, g and h, 19 and 17 cycles were used for the amplification of *Hd3a* and *ubq*, respectively. For real-time PCR used to obtain the results shown in Fig. 3i, quantitative analysis of gene expression was performed by SYBR Green PCR master mix (Applied Biosystems Japan, Tokyo, Japan) with the gene-specific primers listed in Supplementary Table 1. Data were collected using the ABI PRISM 7700 sequence detection system in accordance with the instruction manual.

RNase protection assay

RNase protection assays were performed with the RPA III kit (Ambion, Austin, Texas, USA). Antisense RNA probes for *OsGI*, *Hd1* (*Se1*) and *ubq* were synthesized with T3 or SP6 RNA polymerase (Promega, Madison, Wisconsin, USA). The relative radioactivity of the *Hd1* (*Se1*) probe was adjusted by increasing the concentration of [ $\alpha$ -<sup>32</sup>P]UTP and decreasing the concentration of unlabelled UTP in their reaction cocktails. A total of 30,000 c.p.m. of the purified RNA probe was added in a reaction buffer containing 20 μg of total RNA and annealed overnight with RNA; protected RNA probes were detected on 4% polyacrylamide gel with BAS 2000 (Fuji Photo Film, Tokyo, Japan). The gel image was analysed with MacBAS version 2.52 software to calibrate the signal intensity of each clone. The sizes of the protected RNAs were 83 bp for *OsGI*, 401 bp for *Hd1* (*Se1*) and 245 bp for *ubq*.

Transfection assays in rice protoplasts

Protoplasts (4 × 10<sup>7</sup>) were prepared from the rice Oc cell cultures, mixed with 30 μg of plasmid DNA containing *Hd3a::gus* and 10 μg of plasmid DNA containing *Ubiq::Hd1* (*Se1*) cDNA or *Ubiq::bar* and electroporated with a Gene Pulser (Bio-Rad). A 1.7-kb promoter region of the *Hd3a* gene was used to construct *Hd3a::gus*; it included an intron<sup>23</sup> to enhance *gus* expression. After incubation of protoplasts for 48 h at 30 °C in light, they were harvested and assayed for GUS activity.

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Amino-acid cycling drives nitrogen fixation in the legume–*Rhizobium* symbiosis

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The biological reduction of atmospheric N<sub>2</sub> to ammonium (nitrogen fixation) provides about 65% of the biosphere's available nitrogen. Most of this ammonium is contributed by legume–rhizobia symbioses<sup>1</sup>, which are initiated by the infection of legume hosts by bacteria (rhizobia), resulting in formation of root nodules. Within the nodules, rhizobia are found as bacteroids, which perform the nitrogen fixation: to do this, they obtain sources of carbon and energy from the plant, in the form of dicarboxylic acids<sup>2,3</sup>. It has been thought that, in return, bacteroids simply provide the plant with ammonium. But here we show that a more complex amino-acid cycle is essential for symbiotic nitrogen fixation by *Rhizobium* in pea nodules. The plant provides amino acids to the bacteroids, enabling them to shut down their ammonium assimilation. In return, bacteroids act like plant organelles to cycle amino acids back to the plant for asparagine synthesis. The mutual dependence of this exchange prevents the symbiosis being dominated by the plant, and provides a selective pressure for the evolution of mutualism.

One of the most important aspects of legume–rhizobia symbioses is that bacteroids shut down ammonium assimilation. This could not be explained, although it is considered essential for the evolution of symbioses<sup>4</sup>. However, if plants provide an amino acid to bacteroids, then bacteroids need not assimilate ammonium<sup>5</sup>; instead, they would have to secrete ammonium to the plant to ensure their own amino-acid supply. It has been shown that when isolated peribacteroid units of peas are incubated with a dicarboxylate, addition of glutamate stimulates secretion of aspartate and alanine<sup>6,7</sup>. Although this demonstrates the biochemical capacity for amino-acid cycling, the importance of this to nodule metabolism was hitherto unknown.

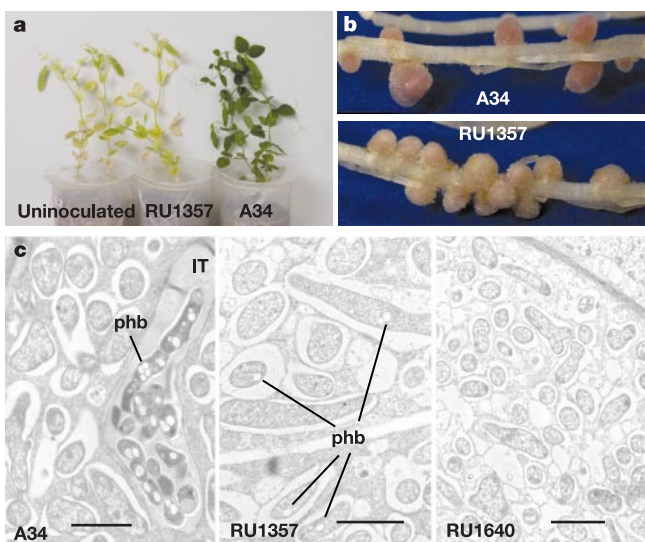
To determine whether amino-acid cycling occurs in pea bacteroids *in planta*, we mutated *aap* and *bra*, which both encode ABC-type broad specificity amino-acid transporters, in *Rhizobium leguminosarum* bv. *viciae*<sup>8–10</sup>. Whereas single mutations in *aap* or *bra* reduced the uptake rates of all tested amino acids by 40–70% in free-living bacteria, the double mutant, RU1357, was almost totally blocked for the uptake of a broad range of amino acids, including glutamate, aspartate and leucine<sup>8</sup>. The double mutant grows on minimal medium, demonstrating that it synthesises amino acids. Whereas the growth of peas nodulated by either *aap* or *bra* single mutants, or by the wild type (A34), was indistinguishable, peas nodulated by RU1357 progressively yellowed (Fig. 1a). The marked reductions in shoot dry weight and shoot nitrogen content of peas nodulated by RU1357, as well as their increased nodule number and mass, are typical of plants unable to fix nitrogen (Fig. 2a–d). These effects can be attributed to the loss of amino-acid transport, as RU1357 carrying the *aap* operon on a stable plasmid (pRU1134) restored plants to normal growth.

Although nitrogen starvation of plants nodulated by RU1357 was very marked, the root nodules were pink, unlike the white nodules usually induced by classical non-fixing mutants, whereas wild-type nodules were deep red (Fig. 1b). The red colour is due to leghaemoglobin, a plant-made O<sub>2</sub>-carrying protein, which is induced by RU1357 at a level intermediate between the wild type and a classical non-fixing mutant. The bacteroid yield from plants nodulated by RU1357 was 37% of that of A34. Acetylene and <sup>15</sup>N<sub>2</sub> reduction

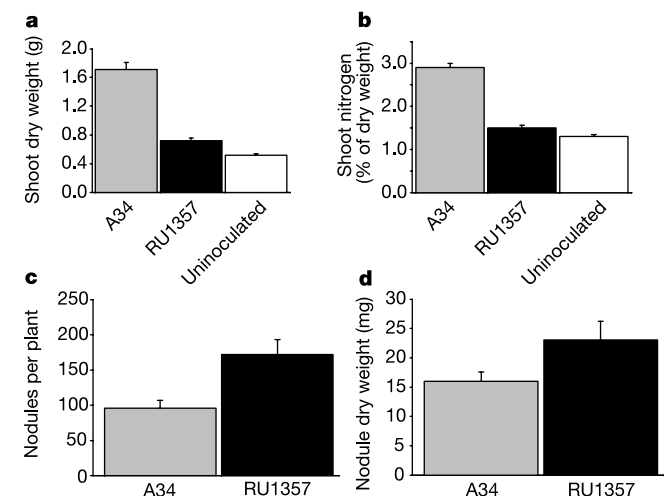
assays showed that plants nodulated by RU1357 fixed nitrogen at 32–55% of wild-type rates (Fig. 3a, c). When these rates are expressed on a bacteroid protein basis, the values for RU1357 are similar (<sup>15</sup>N<sub>2</sub> reduction) or even higher (acetylene reduction) than the wild type (Fig. 3b, d). Isolated bacteroids of RU1357 reduced <sup>15</sup>N<sub>2</sub> at substantially higher rates than A34 (Fig. 3e, f). This shows that RU1357 bacteroids, blocked for amino-acid transport, can reduce N<sub>2</sub>, but apparently the plant cannot acquire the resulting ammonium. This is in disagreement with our current models of both bacterial and plant-nodule metabolism.

We examined directly the fate of <sup>15</sup>N<sub>2</sub> fixed in nodules by measuring xylem amide levels and <sup>15</sup>N enrichment. The concentrations of xylem amides were extremely low in plants nodulated by RU1357 compared to A34 (Fig. 3g), with the concentration of asparagine for RU1357 only 11% of that for A34. Correcting the xylem amide concentration for bacteroid protein still leaves a low asparagine level for plants nodulated by RU1357 relative to the wild type (Fig. 3h). This is in contrast to ammonium production per unit bacteroid protein, which is similar between plants nodulated by A34 or RU1357 (Fig. 3d). As expected, there was high <sup>15</sup>N enrichment of glutamine in the xylem sap of plants nodulated by A34, but also of RU1357 (Fig. 3i). Asparagine was also significantly enriched in <sup>15</sup>N in plants nodulated by both A34 (0.82 atom% excess) and RU1357 (0.33 atom% excess), although predictably at lower levels than glutamine (Fig. 3i). These <sup>15</sup>N enrichments show that plants retain the biochemical capacity to make amides. However, in the absence of amino-acid transport by bacteroids, plants cannot efficiently use the ammonium released, resulting in the extremely low amide concentration. An explanation is that bacteroids provide both ammonium and an amino acid such as aspartate for asparagine synthesis in the plant cytosol.

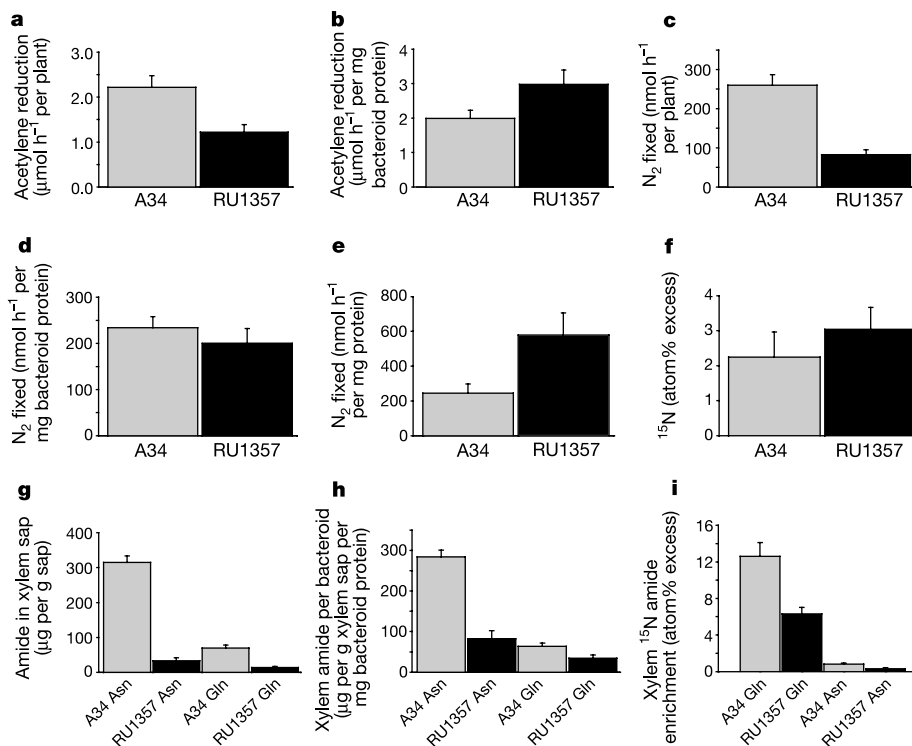
It is widely accepted that bacteroids accumulate dicarboxylates (fumarate, succinate or L-malate) via the dicarboxylic-acid transport system, and that these are oxidized to provide energy for N<sub>2</sub> reduction to ammonium<sup>11,12</sup>. We propose a model in which glutamate (or a precursor to it) is transported into bacteroids, in addition to dicarboxylates (Fig. 4). The glutamate would enter via *Aap/Bra*, and act as a transamination donor to produce aspartate, or possibly amino acids such as alanine. These would be secreted to the plant enabling asparagine synthesis. Glutamate is the most probable donor amino acid, because it is highly abundant in nodules and is known to stimulate transamination of oxaloacetate and pyruvate in



**Figure 1** Effect of mutation of amino-acid uptake in *R. leguminosarum* on growth and nodulation of peas. **a**, Peas (*Pisum sativum* c.v. Avola) were left uninoculated, inoculated with *R. leguminosarum* strain RU1357 (*aap/bra*) or A34 (wild type) and grown for 40 d on nitrogen-free rooting solution. **b**, Three-week-old nodules of A34 and RU1357. **c**, Electron micrographs of plant cells infected with A34, RU1357 or RU1640 (*aat4*). Scale bars, 2  $\mu$ m. phb, polyhydroxybutyrate granule; it, infection thread.



**Figure 2** Growth and nodulation of peas infected by either wild type or a mutant unable to transport amino acids. **a**, Shoot dry weight ( $n = 22$  plants). **b**, Shoot nitrogen as a percentage of dry weight ( $n = 8$  plants). **c**, Nodule number per plant ( $n \geq 7$  plants). **d**, Nodule dry weight per plant ( $n \geq 7$  plants). All data shown  $\pm$  s.e.m.



**Figure 3** Nitrogen fixation and assimilation of peas infected by either wild type or a mutant unable to transport amino acids. **a**, Acetylene reduction by plants expressed per plant ( $n \geq 12$  plants). **b**, Acetylene reduction by plants expressed per unit bacteroid protein ( $n \geq 12$  plants). **c**,  $N_2$  fixed by plants expressed per plant ( $n \geq 9$  plants). **d**,  $N_2$  fixed by plants expressed per unit bacteroid protein ( $n \geq 9$  plants). **e**, Total  $N_2$  reduced to

ammonium by isolated bacteroids ( $n \geq 3$  assays). **f**,  $^{15}N$  isotope enrichment of ammonium produced by isolated bacteroids ( $n \geq 3$  assays). **g**, Xylem amide concentration per plant ( $n = 9$  plants). **h**, Xylem amide concentration expressed per unit bacteroid protein ( $n = 9$  plants). **i**,  $^{15}N$  enrichment of pea xylem amides ( $n = 9$  plants). All data shown  $\pm$  s.e.m.

isolated pea peribacteroid units, leading to aspartate and alanine secretion<sup>6,7</sup>. As Aap and Bra have a broad solute specificity, amino acids other than glutamate may be taken up from the plant. However, most bacterial amino-acid metabolism is channelled via glutamate, making little difference between direct uptake of glutamate or a precursor. Aspartate is the most likely secretion product, because glutamate stimulates its synthesis in isolated peribacteroid units, and a blockage in its secretion would dramatically reduce asparagine synthesis in the plant. However, the secretion of alanine by bacteroids could have a similar effect, because alanine, glutamate and aspartate can be interconverted in the plant by glutamate pyruvate transaminase and aspartate aminotransferase. We have previously shown that, apart from their role as uptake transporters, Aap and Bra also act as high-rate but low-affinity exporters, and therefore may also mediate the export of aspartate or other amino acids<sup>9,10</sup>. Blocking amino-acid import, export or both will have similar effects.

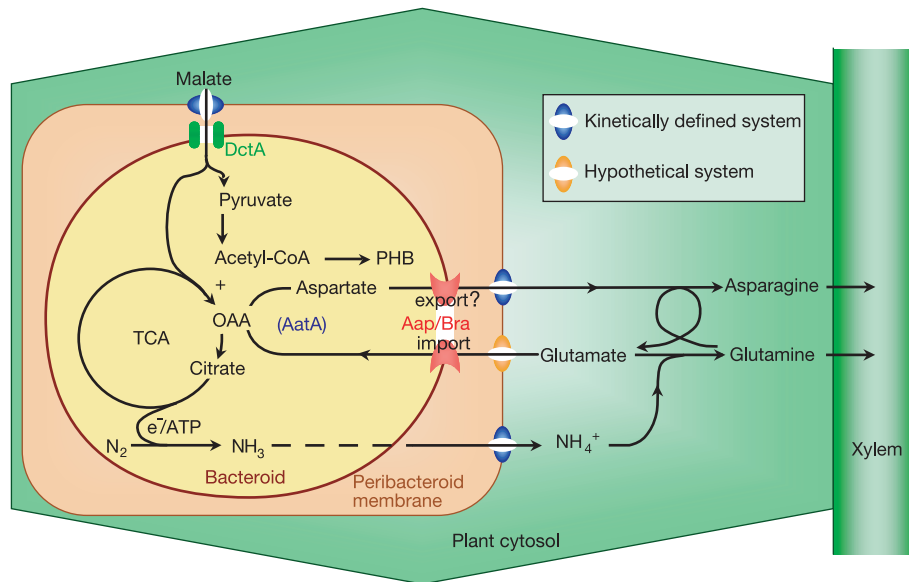
There are two predictions of this model: first, bacteroid aspartate aminotransferase (Aat) activity should be essential for nitrogen fixation; and second, blocking amino-acid transport may cause bacteroids to become carbon saturated, because they can no longer remove dicarboxylic acids via transamination and amino-acid export.

Mutation of the principal aspartate aminotransferase gene (*aatA*) in *Sinorhizobium meliloti* blocks nitrogen fixation by alfalfa<sup>13</sup>. The reason for this was unknown, but it was considered unusual because an *aatA* mutant retained significant levels of Aat activity owing to other secondary aminotransferases. To determine if AatA is also essential for an effective symbiosis in pea, we generated an *aatA* mutant in A34 (RU1640). Strains A34, RU1640 and RU1640/pRU1133 (*aatA* cloned in pJP2) grown on glucose/ammonium minimal medium had Aat activities of  $0.385 \pm 0.113 \mu\text{mol min}^{-1}$

per mg protein (mean  $\pm$  s.e.m.,  $n = 4$ ),  $0.003 \pm 0.003 \mu\text{mol min}^{-1}$  per mg protein ( $n = 3$ ) and  $1.343 \pm 0.427 \mu\text{mol min}^{-1}$  per mg protein ( $n = 3$ ), respectively. Plants inoculated with RU1640 did not fix nitrogen, yellowing from 4 weeks and forming white nodules with no detectable acetylene reduction. Thus, as predicted, aspartate aminotransferase is needed in pea bacteroids for effective nitrogen fixation. Consistent with this, the activity of AatA in A34 bacteroids was high at  $0.162 \pm 0.017 \mu\text{mol min}^{-1}$  per mg protein ( $n = 3$ ).

Electron micrographs of nodule sections show that bacteroids mutated in *aatA* or *aap/bra* are fully developed, and are similar to both wild type and *dct* mutants<sup>11,12</sup> (Fig. 1c). Light micrographs of nodules infected with RU1640 (*aatA*) or RU1357 (*aap/bra*) show a large accumulation of plant starch relative to A34. This accumulation is consistent with either no (RU1640), or lower (RU1357), symbiotic nitrogen fixation and therefore reduced carbon demand on the plant. Infection threads containing A34, RU1357 or RU1640 have undifferentiated bacteria containing electron-transparent polyhydroxybutyrate granules (Fig. 1c). Remarkably, only RU1357 bacteroids contain polyhydroxybutyrate granules. This is consistent with them using dicarboxylates inefficiently, because in the absence of imported glutamate they cannot make aspartate from oxaloacetate (Fig. 4). While A34 bacteroids would secrete aspartate, RU1357 presumably increases carbon flow from the dicarboxylate L-malate to pyruvate via malic enzyme. Pyruvate leads to polyhydroxybutyrate synthesis. The absence of polyhydroxybutyrate granules in *aatA* or *dctA* mutants is consistent with a blockage in dicarboxylate use.

The cessation of  $N_2$  reduction in *aatA* or *dctA* mutants is more extreme than the phenotype of the amino-acid transport mutant RU1357. One possibility is that bacteroids mutated in *aatA* or *dctA* are metabolically disrupted by accumulated glutamate, which cannot be converted to aspartate. The *aap/bra* transport mutant would not accumulate glutamate<sup>8,9</sup>. However, it is possible that *aatA*



**Figure 4** The role of amino-acid cycling in nitrogen fixation in pea nodules. Only reactions directly involved in amino-acid cycling in the bacteroid and plant are shown. Transport systems from the peribacteroid membrane that have been kinetically but not genetically characterized are shown in blue, while those that are hypothetical are in yellow. Although glutamate and aspartate are shown as the amino acids most likely to cycle,

others (such as alanine) may be important. The reaction catalysed by AatA also forms 2-ketoglutarate, which may be either metabolized by the bacteroid or exported back to the plant. Export via Aap/Bra is shown with a question mark to indicate that it is hypothetical. PHB, polyhydroxybutyrate; OAA, oxaloacetic acid; TCA, tricarboxylic acid cycle.

and *dctA* mutants have regulatory effects beyond the prevention of dicarboxylate uptake and transamination. We note that *rug4* mutants (sucrose synthase minus) of pea plants, which cannot provide dicarboxylates to bacteroids, lack classical non-fixing nodules<sup>14</sup>. Instead, they resemble nodules containing *aap/bra* mutants in that they contain nitrogenase and leghaemoglobin, but do not provide significant fixed nitrogen to the plant.

Our model also predicts that the *in planta* effects of amino-acid transport mutations in bacteroids should be confined to ammonium assimilation in the nodule. To test this, pea plants nodulated by RU1357 were grown for three weeks on nitrogen-free medium, eliciting nitrogen starvation, and then either NaNO<sub>3</sub> (5 mM) or NH<sub>4</sub>Cl (5 mM) was added. The plants rapidly greened, and growth recovered. Thus, although plants infected with strain RU1357 are defective in nitrogen assimilation in nodules, they retain the ability to assimilate nitrate or ammonium supplied to roots.

Also consistent with our model is the demonstration that glutamate stimulates aspartate and alanine secretion by isolated pea bacteroids<sup>6,7</sup>. These studies do have inconsistent stoichiometries between solutes accumulated and secreted, but recently it was shown that isolated pea bacteroids are severely damaged, with cytoplasmic and periplasmic proteins incorrectly found in the peribacteroid space<sup>15</sup>. In particular, the AapJ and BraC periplasmic binding proteins were in the peribacteroid space, which would inactivate transport by Aap/Bra. Thus, studies with isolated bacteroids are unlikely to give rates of flux or stoichiometries that are the same as inside the nodule. The demonstration that isolated pea peribacteroid units secrete aspartate and alanine, when given malate and glutamate, was done using *R. leguminosarum* strain 3841, rather than A34 used in this study<sup>6</sup>. To enable comparison between these studies we generated an *aap/bra* mutant in strain 3841. Peas inoculated with either RU1722 (3841 *aap/bra*) or RU1357 were indistinguishable with regard to plant growth.

The permeability of the peribacteroid membrane to amino acids and organic acids is critical to the operation of any amino-acid cycle (Fig. 4). The pea peribacteroid membrane has an ammonium channel and a H<sup>+</sup>/aspartate export system that move solutes from

the peribacteroid space into the plant cytosol<sup>16,17</sup>. These systems are essential to our model. A dicarboxylate transport system has been characterized in the peribacteroid membrane of soybeans, and a similar system is probably present in all peribacteroid membranes<sup>18</sup>. However, active glutamate uptake across peribacteroid membranes has not been detected<sup>19</sup>. Possibly, like bacteroid membranes, isolated peribacteroid membranes are damaged or incorrectly energized, and there is a loss of detectable glutamate transport. Alternatively, either glutamate moves passively or another amino acid moves across the peribacteroid membrane and is transported into bacteroids by Aap/Bra.

There are significant consequences of this model for the legume–*Rhizobium* symbiosis. As the plant provides bacteroids with amino acids, bacteroids can shut down ammonium assimilation. To obtain amino acids, bacteroids must secrete ammonium to the plant, enabling amino-acid synthesis. This provides a powerful selective pressure for the evolution of symbiosis, and suggests that the plant can regulate bacteroid dicarboxylate use by amino-acid supply. This could lead to the plant dominating the symbiosis. However, bacteroids act like plant organelles for aspartate synthesis, making the plant dependent on them. This provides a counter selection to plant dominance, and favours the evolution of mutualism. However, it is not a classical malate/aspartate shuttle, which transfers reductant but not carbon into mitochondria<sup>20</sup> and has been suggested might operate in bacteroids<sup>5</sup>. Bacteroids of RU1357 reduce N<sub>2</sub> to ammonium at specific rates similar to wild type, demonstrating they generate adequate reductant. The role of amino-acid cycling is to facilitate both dicarboxylate oxidation and ammonium assimilation into asparagine. The interaction between the symbiotic partners is far more complex than hitherto realized: each has evolved a complete metabolic dependence on the other. □

## Methods

Strains A34 (wild type) and RU1357 ( $\Delta aapJQM::\Omega Sp\ braE::TnphoA$ ) were grown on acid minimal medium<sup>8,21</sup>. An *aap/bra* double mutation (RU1722  $\Delta aapJQM::\Omega Sp\ \Delta braEF::\Omega Tc$ ) was made in strain 3841. RU1722 has the  $\Delta aapJQM::\Omega Sp$  mutation as in RU1357, but the *bra* system was mutated by deleting the *EcoRV* fragment that spans part of

*braE* and *braF*. Peas (*Pisum sativum* c.v. Avola and Winner) were grown in 2-l pots in vermiculite with nitrogen-free rooting solution<sup>21</sup>. Shoot dry weights were determined on 6-week-old plants by drying for 48 h at 70 °C, and total nitrogen determined by Dumas combustion. All other experiments on plants or bacteroids were determined with 3–4-week-old plants. Optical and electron-micrograph images were determined on sectioned nodules<sup>22</sup>. Total bacteroid protein per plant was determined by removing all nodules from plants, isolating bacteroids by Percoll purification<sup>21</sup> and determining protein by the Lowry method. Acetylene reductions were determined on plants incubated in 95% air 5% acetylene for 1 h in 250-ml Schott bottles<sup>21</sup>. To measure <sup>15</sup>N<sub>2</sub> reduction, plants were incubated in 80% air 20% <sup>15</sup>N<sub>2</sub> (98 atom%, Isotec) for 1 h in 250-ml Schott bottles. The incorporation of <sup>15</sup>N<sub>2</sub> was determined by separating roots and shoots, grinding them in 3–4 ml of HCl (0.1 M), drying overnight and measuring total nitrogen and <sup>15</sup>N enrichment in a continuous flow isotope ratio mass spectrometer (TracerMat, Finnigan MAT)<sup>21</sup>. Synthesis of amino acids in pea xylem was measured in plants incubated as for total <sup>15</sup>N<sub>2</sub> reduction, except that the incubation time was 30 min. Xylem sap was removed from the 3-cm stem section immediately below the first leaf<sup>23</sup>. The concentrations and <sup>15</sup>N enrichment of amino acids were determined with a Trace 2000 gas chromatograph (Finnigan) fitted with an AS 2000 autosampler (Finnigan) and interfaced to a Trace quadrupole mass spectrometer (Finnigan)<sup>21</sup>. Bacteroids were isolated in a microaerobic cabinet (MACS-MG-1000, Don Whately), under an atmosphere of 0.1% O<sub>2</sub>, 1% CO<sub>2</sub> and 98.9% N<sub>2</sub>, and <sup>15</sup>N<sub>2</sub> reduction to ammonium was determined using a Roboprep combustion analyser (Europa Scientific) interfaced to a VG 622 isotope ratio mass spectrometer<sup>21</sup> (VG).

The *R. leguminosarum aata* gene was sequenced (EMBL AJ006709), mutated and recombined into strain A34 to create RU1640. The Gene Jumper kanamycin cassette (Invitrogen) in *aata* is located between the 5-base-pair duplication of bases 409–413 (GGCAC), and its recombination into A34 to create strain RU1640 was confirmed by Southern blotting. The *aata* gene was amplified by polymerase chain reaction with primers p383 (AGGTGACGCAAGCCTCTCC) and p384 (ACCGTCACTCTGCCGTCACG), and cloned as an *Xba*I/*Bam*HI fragment into the stable broad host range plasmid pJP2<sup>24</sup>, creating pRU1133. The *aap*JQMP operon was cloned from pRU189<sup>10</sup> as an *Xba*I/*Hind*III fragment into pJP2, creating pRU1134.

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## Transcription-targeted DNA deamination by the AID antibody diversification enzyme

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Activation-induced cytidine deaminase (AID), which is specific to B lymphocytes, is required for class switch recombination (CSR)—a process mediating isotype switching of immunoglobulin—and somatic hypermutation—the introduction of many point mutations into the immunoglobulin variable region genes<sup>1,2</sup>. It has been suggested that AID may function as an RNA-editing enzyme<sup>3</sup> or as a cytidine deaminase on DNA<sup>4,5</sup>. However, the precise enzymatic activity of AID has not been assessed in previous studies. Similarly, although transcription of the target immunoglobulin locus sequences is required for both CSR and somatic hypermutation, the precise role of transcription has remained speculative<sup>6–9</sup>. Here we use two different assays to demonstrate that AID can deaminate specifically cytidines on single-stranded (ss)DNA but not double-stranded (ds)DNA substrates *in vitro*. However, dsDNA can be deaminated by AID *in vitro* when the reaction is coupled to transcription. Moreover, a synthetic dsDNA sequence, which targets CSR *in vivo* in a manner dependent on transcriptional orientation<sup>10</sup>, was deaminated by AID *in vitro* with the same transcriptional-orientation-dependence as observed for endogenous CSR. We conclude that transcription targets the DNA deamination activity of AID to dsDNA by generating secondary structures that provide ssDNA substrates.

Immunoglobulin heavy (IgH) and light (IgL) chain variable region exons are assembled in developing B-lineage cells by recombination of V, D and J segments—V(D)J recombination<sup>11</sup>. After antigen-dependent activation, mature B cells undergo additional immunoglobulin locus alterations, namely CSR and somatic hypermutation<sup>6–9</sup>. CSR changes the expressed IgH constant region exons (C<sub>H</sub>) from C<sub>μ</sub> to a downstream C<sub>H</sub> (for example, C<sub>γ</sub>, C<sub>ε</sub>, C<sub>α</sub>), allowing association of the variable region with different C<sub>H</sub> effector functions<sup>6,7</sup>. CSR occurs between 1–12-kilobase (kb) repetitive switch (S) region sequences located 5' of each C<sub>H</sub>. S regions are required for efficient CSR<sup>10,12</sup>, as is transcription through an S region before CSR<sup>6,13</sup>. In this regard, a 1-kb synthetic sequence that generates a G-rich transcript, but that lacks S region motifs, effects CSR in a transcriptional-orientation-dependent manner in place of S<sub>γ1</sub> *in vivo*<sup>10</sup>. This finding suggested that ssDNA that is stabilized by transcription-dependent higher-order structures might be a