出典： Nature 462, 514-517 (26 November 2009)
DOI： 10.1038/nature08594
リンク先： http://dx.doi.org/10.1038/nature08594

Host plant genome overcomes the lack of a bacterial gene for symbiotic nitrogen fixation

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Homocitrate is a component of the iron-molybdenum cofactor (FeMo-cofactor) in nitrogenase, where nitrogen fixation occurs[1,2]. *NifV*, which encodes homocitrate synthase (HCS)[3], has been identified from various diazotrophs, but is not present in most of rhizobium species that exert efficient nitrogen fixation only in symbiotic association with legumes. Here we show that the *FEN1* gene of a model legume, *Lotus japonicus*, overcomes the lack of *NifV* in rhizobia for symbiotic nitrogen fixation. A Fix$^-$ plant mutant, *fen1*, forms morphologically normal but ineffective nodules[4,5]. The causal gene, *FEN1*, was shown to encode HCS by its ability to

complement a *Saccharomyces cerevisiae* HCS-defective mutant. Homocitrate was present abundantly in wild-type nodules but was absent in ineffective *fen1* nodules. Inoculation with *Mesorhizobium loti* carrying *FEN1* or *Azotobacter vinelandii NifV* rescued the defect in nitrogen-fixing activity of the *fen1* nodules. Exogenous supply of homocitrate also recovered the nitrogen-fixing activity of the *fen1* nodules through *de novo* nitrogenase synthesis in the bacteroids. These results indicate that homocitrate derived from the host plant cells is essential for the efficient and continuing synthesis of nitrogenase system in endosymbionts, and thus provides a molecular basis for the complementary and indispensable partnership between legumes and rhizobia in symbiotic nitrogen fixation.

The major source of nitrogen for all living organisms is atmospheric dinitrogen, which is mainly fixed by microorganisms that have an ability to reduce dinitrogen to ammonium by a nitrogenase system. In legume plants, soil bacteria of the family *Rhizobiaceae* (rhizobia) are hosted within a symbiotic organ, the root nodule, in which the endosymbiotic rhizobia are able to fix dinitrogen. This enables the host legumes to grow without an exogenous nitrogen source. Unlike many free-living diazotrophs, rhizobia are able to exhibit highly efficient nitrogen fixation only when they are in the host nodule cells as an endosymbiotic form, the bacteroid. This indicates that rhizobial nitrogen fixation is controlled by the host plant. Fix^- mutants of the host legumes that form ineffective nodules are key tools to identify the host genes essential for establishment of symbiotic nitrogen fixation.

A *L. japonicus* Fix^- mutant, *fen1*[4,5], forms small, pale-pink nodules and displays nitrogen deficiency symptoms under symbiotic conditions (Supplementary Fig. 1a-c and f-h). In the *fen1* nodules, rhizobial invasion of the nodule cells appeared to be comparable to wild-type Gifu (Supplementary Fig. 1d and e), but the nitrogenase activity remained at very low levels (Supplementary Fig. 1i and j).

We identified the responsible gene, *FEN1*, for the *fen1* mutant through map-based cloning, and confirmed the complementation of the mutant phenotypes by *Agrobacterium rhizogenes*-mediated hairy root transformation (Supplementary Results and Supplementary Fig. 2). Transcripts of *FEN1* were detected only in nodules, indicating that expression of the *FEN1* gene was regulated in a nodule specific manner (Fig. 1a). When the *FEN1* promoter-ß-glucuronidase (GUS) fusion was introduced, GUS activity was detected only in infected cells of nodules (Fig. 1b and c). By searching *L. japonicus* EST database[6], we found a paralogous clone to the *FEN1* gene, MWM049f12, of which predicted amino-acid sequence had 91% identity to that of the *FEN1* gene. However, expression of MWM049f12 was detected in all organs of *L. japonicus* at low levels, and was not enhanced in nodules. These results indicate that FEN1 is closely associated with nitrogen-fixing activity of the nodules.

The predicted FEN1 protein consisted of 540 amino acids with a molecular mass of 58,600. Any signal peptide sequences were not found, suggesting that it is a cytosolic protein. Deduced amino-acid sequence of FEN1 had 71% identity to that for the *Glycine max* nodule-specific gene, *GmN56*. The introduction of *GmN56* cDNA into the *fen1* mutant recovered growth and nitrogenase activity of the mutant (Supplementary Fig. 3a and b), indicating that *GmN56* is an ortholog of the *FEN1* gene. *GmN56* has been shown to be induced with the onset of nitrogen fixation and the transcripts are localized in the bacterial infected cells of mature nodules of soybean[7], consistent with the expression pattern of the *FEN1* gene in *L. japonicus* nodules. The predicted GmN56 protein showed homology to 2-isopropylmalate synthase (IPMS) and homocitrate synthase (HCS), though the exact function of the GmN56 protein has not been confirmed. Besides *GmN56*, several genes encoding IPMS isolated from plants such as *Brassica atlantica*, *Arabidopsis thaliana*, and *Lycopersicon pennellii* were found to show high similarity (around 66% amino acid sequence identity) with FEN1.

To explore the function of FEN1, we first introduced the *FEN1* gene into a *S. cerevisiae* IPMS defective mutant[8]. *FEN1* failed to complement leucine auxotrophy of the *S. cerevisiae* mutant, and the cell extract exhibited no IPMS activity (Supplementary Fig. 4a and b). By contrast, we detected substantial activity of IPMS in the extract of *S. cerevisiae* transformed with the *Arabidopsis* IPMS2 (At1g74040) gene[9] and the complementation of leucine auxotrophy, even though in part (see the legend for Supplementary Fig. 4). In addition, the Fix^- phenotype of *fen1* was not recovered by introduction of the *AtIPMS2* gene (Supplementary Fig. 3a and b). From these results, we concluded that the *FEN1* gene does not code for IPMS.

We next focused on HCS, which catalyzes the synthesis of homocitrate from 2-oxoglutarate and acetyl-CoA. IPMS and HCS are different enzymes, but they have some structural similarity[7]. They both catalyze similar reactions; the transfer of an acyl-group from acetyl-CoA to 2-oxo acid to generate the alkyl-group in 2-oxo acid. The FEN1 protein has 36% identity to HCS (NIFV) of the nitrogen-fixing aerobic bacteria *Azotobacter vinelandii*[3]. We introduced the *FEN1* gene into a *S. cerevisiae* mutant which shows lysine auxotrophy caused by the lack of HCS[10]. The introduction of the *FEN1* gene, but not *Arabidopsis IPMS2* and mutated *FEN1* gene, complemented lysine auxotrophy of the mutant (Fig. 2a). Furthermore, significant accumulation of homocitrate was found in the transformed *S. cerevisiae* mutant when expression of the *FEN1* gene was induced (Supplementary Fig. 5). These results demonstrated that the recombinant FEN1 protein confers HCS activity. In the present study, we were unable to detect HCS activity *in vitro* in cell-free extracts of *Lotus* nodules. We thus investigated the presence of homocitrate in various tissues of *L. japonicus* to confirm HCS activity *in vivo*. LC/MS/MS analysis showed that, in wild-type Gifu plants, homocitrate was detected abundantly in nodules, but neither in roots nor shoots (Fig. 2b). By contrast, it was barely detectable (less than 1% of wild-type nodules) in ineffective nodules formed on the *fen1* mutant (Fig. 2c). The ineffective nodules formed

by the *NifH* defective mutant of *M. loti* contained homocitrate at the level comparable to that in the wild-type nodules, indicating that accumulation of homocitrate in nodules is not the result of active nitrogen fixation. In addition, the level of 2-oxoglutarate was found to be higher in the *fen1* nodules than the wild-type nodules and ineffective nodules formed by the *NifH* defective mutant of *M. loti* (Fig. 2c). These results indicate that *FEN1* encodes HCS and the activity is lost in nodules of the *fen1* mutant.

In higher plants, a metabolic pathway leading to synthesis of lysine through homocitrate as an intermediate has not been identified. Here we took notice of the fact that homocitrate is a component of the FeMo-cofactor of nitrogenase complex in nitrogen-fixing bacteria[2]. Therefore, homocitrate synthesized in host plant cells was expected to be transported to bacteroids and utilized for biosynthesis of the nitrogenase complex. We examined this hypothesis by introducing the *FEN1* gene into *M. loti* under the control of the rhizobial *NifH* promoter. Inoculation with *M. loti* carrying the *FEN1* gene to the *fen1* mutant rescued either the defect in nodule nitrogenase activity or the plant growth (Fig. 3a and b). Expression of FEN1 in the bacteroids of nodules formed by transformed *M. loti* was confirmed by immuno-detection of FEN1-myc fusion protein (Fig. 3c). In a similar way, we tested inoculation with *M. loti* carrying the *A. vinelandii NifV* gene, which has been well demonstrated to encode HCS and to be essential for nitrogenase activity[3]. *M. loti* with expression of *A. vinelandii NifV* could also rescue the *fen1* mutant phenotypes (Fig. 3d-f). Furthermore, we found that addition of homocitrate into the culture solution restored in part the effectiveness of the nodules formed on the *fen1* mutant (Fig. 4a and b). In the *fen1* nodules supplied with homocitrate, amounts of nitrogenase proteins were recovered to the level nearly comparable to those in the wild-type nodules (Fig. 4c), indicating that the restoration of nitrogenase activity by the supply of homocitrate was due to *de novo* nitrogenase biosynthesis. Taken together, these results indicate that rhizobial nitrogen-fixing activity depends on the homocitrate derived from the host plant, which could be utilized for

assembly of the FeMo-cofactor in nitrogenase complex in the endosymbionts (Supplementary Fig. 6).

Rhizobial nitrogen-fixing activity is restricted to symbiotic bacteroids, and free-living rhizobia normally fix no atmospheric nitrogen, representing a unique feature of the legume/*Rhizobium* symbiosis[11]. This could be explained in part by the fact that *NifV* gene encoding HCS[3] has not been identified in most rhizobia, except in stem nodulating rhizobia, *Azorhizobium caulinodans,* and in photosynthetic rhizobia, *Bradyrhizobium* sp. strains[12]. *Azorhizobium* has been shown to fix atmospheric nitrogen in culture[13]. In addition, the photosynthetic rhizobia have distinctive features including the absence of nodulation genes, and have been proposed to belong to a distinct group in *Rhizobiaceae*[14,15]. In other nitrogen-fixing symbiotic associations, however, the *NifV* gene was identified in three types of microsymbionts: *Frankia, Anabaena,* and some endophytic bacteria[12], which are capable of fixing nitrogen in a free-living state[16]. Our results, together with these previous observations, led us to the idea that the absence of the *NifV* gene in rhizobia is compensated by the *FEN1* gene in the host legume genome to acquire highly efficient nitrogen fixation in symbiosis. Nevertheless, some strains of rhizobia can fix nitrogen at free-living state, even though not efficiently in most cases, under defined conditions[17,18,19], and ineffective nodules induced on the *fen1* mutant exhibited a low rate of nitrogen-fixing activity. Therefore, rhizobia are likely able to synthesize homocitrate by alternative pathway(s) without *NifV*. Alternatively, citrate may be, in part, substituted for homocitrate in the FeMo-cofactor, as reported for *Klebsiella pneumoniae*[20].

We found, for the first time, a gene encoding HCS in the higher plant kingdom and demonstrated that homocitrate supply from the host plant cells to endosymbiotic bacteroids is essential for symbiotic nitrogen fixation. The FEN1 protein exhibited a higher structural similarity to plant IPMSs rather than bacterial HCSs, but this could be

reflected in the species difference between higher plants and microorganisms. Indeed, FEN1 had only about 40% similarity with bacterial IPMSs such as LEUA of *Escherichia coli*. It should be also noted that the IPMS has shown to be capable of using various 2-oxo acids as the substrates[9,21,22]. It is thus very likely that the *FEN1* gene had been recruited from a house-keeping IPMS gene during the evolution of symbiosis, and made it possible to establish efficient nitrogen fixation by endosymbiotic bacteria. Such recruitment has been also suggested for a number of nodule-specific (nodulin) genes, such as leghemoglobins[23], uricase[24], and phosphoenolpyruvate carboxylase[25]. Furthermore, metabolic partnerships between host legumes and microsymbionts have been well documented. Supply of dicarboxylates and amino acids from the host cells to bacteroids has been shown essential for nitrogen fixation and/or differentiation of the bacteroids[26,27]. However, our finding is distinguished from those previous ones by two aspects. The first is that FEN1 has developed a function as a HCS, which is distinct from IPMS, and has not been found so far in higher plants. The second is that FEN1 produces homocitrate, which is an essential component of the nitrogenase complex, but is not required *per se* for plant metabolism, and thus could compensate the lack of *NifV* in rhizobia. Our data support the idea that the acquirement of HCS by the nodule specific *FEN1* gene in host legumes constituted one of the key genetic inventions for the establishment of a highly efficient nitrogen-fixing symbiosis by legumes and rhizobia, thus providing a new insight into co-evolution of metabolic pathways between two symbiotic partners.

METHODS SUMMARY

The plant-determined Fix$^-$ mutants *fen1-1*[4,5] and *fen1-2* were derived from *L. japonicus* accession B-129 “Gifu” and accession MG-20 “Miyakojima”, respectively, by ethylmethane sulfonate (EMS) mutagenesis. *M. loti* strain MAFF303099, TONO, and *NifH*-defective mutant of MAFF303099 were used for inoculation. Map-based cloning

of the *FEN1* gene was carried out by the cross of the *fen1-1* mutant with MG-20, using SSR and dCAPS markers[28,29], together with additionally developed PCR-based markers. Since two *fen1* mutant alleles, *fen1-1* and *fen1-2*, showed essentially the same phenotypes, the *fen1-1* mutant was used for all further analyses. The *FEN1*, *GmN56*, and *AtIPMS2* constructs (p*FEN1*-cDNA-t*FEN1*) were introduced into the *fen1* mutant by *A. rhizogenes*-mediated hairy root transformation. Functional complementation of *S. cerevisiae* mutants was performed with pYES2 yeast expression vector containing *GAL1* promoter (Invitrogen). Carboxylic acid fractions were prepared from nodules, roots and shoots with ion exchange resin. Amounts of 2-oxoglutalate and homocitrate were analyzed by anion-exclusion HPLC and LC/MS/MS, respectively. Authentic homocitrate standard was synthesized as described[30]. The p*NifH-FEN1*-3x *myc* or p*NifH-A. vinelandii NifV*-3x *myc* fragment was inserted into the transposon plasmid, pCAM120, followed by transfection into *M. loti* TONO by tri-parental mating, which were successively inoculated to the wild-type and the *fen1* mutant plants. Synthetic homocitrate was supplied to the *fen1* mutant by immersing the nodulated roots in the culture solution containing 1 mM homocitrate.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank E. Casalone and E. Dubois for providing *Saccharomyces cerevisiae* mutants; T. Bisseling for providing nitrogenase antibody; Y. Kawaharada and H. Mitsui for technical

help; R.W. Ridge for critical reading of the manuscript. This work was supported by the Special Coordination Funds for Promoting Science and Technology of the Japanese Ministry of Education, Culture, Sports, Science and Technology.

Author Contributions All authors contributed extensively to the experimental works. The manuscript was written by T.H., H.K. and N.S.

Author Information The sequences have been deposited at the DNA Data Bank of Japan with the following accession numbers: LjT09C23 (AP004466), LjT02F04 (AP010267), LjT28C03 (AP010268) and mRNA sequence (Gifu B-129) of *FEN1* (AB494481). Reprints and permissions information is available at www.nature.com/reprints. The authors declare that they have no competing financial interests. Corresponding and requests for materials should be addressed to N.S. (nsuganum@auecc.aichi-edu.ac.jp).

Figure 1 Expression analyses of the *Lotus japonicus FEN1* gene. **a**, Northern blot analysis of the *FEN1* and the homologous *MWM049f12* transcripts in various organs (left) and during nodule development (right). Ribosomal RNA (rRNA) was stained with ethidium bromide. **b**, **c**, Spatial expression analysis of the *FEN1* gene in the wild-type nodules by *FEN1* promoter-GUS fusion. Transgenic hairy roots were inoculated with *Mesorhizobium loti* TONO, and the sections of nodules with the *FEN1* promoter-GUS (**b**) and with empty vector (**c**) were examined for histochemical GUS assay. Positive signals are visible as blue colour development. Scale bars = 200 μm.

Figure 2 Functional complementation of a *Saccharomyces cerevisiae* homocitrate synthase defective mutant, and homocitrate and 2-oxoglutarate content in nodules. **a**, Complementation test of *S. cerevisiae* mutant by *Lotus japonicus FEN1* gene. *S. cerevisiae* mutants carrying empty vector (EV), *FEN1*,

mutated *FEN1* (*FEN1m*, corresponding to mutation in *fen1-1*), and *Arabidopsis* isopropylmalate synthase (*AtIPMS2*) were grown on medium with (+) or without (–) lysine (Lys). **b**, Homocitrate content in nodules, shoots and roots of the wild-type Gifu inoculated with *Mesorhizobium loti* TONO. **c**, Homocitrate and 2-oxoglutarate content in nodules of the wild-type Gifu and *fen1-1* mutant inoculated with *M. loti* TONO, and nodules induced by *M. loti NifH* mutant. The data are means of two independent nodulated roots with standard errors.

Figure 3 Complementation of the *fen1* mutants by inoculation with *Mesorhizobium loti* carrying *NifH* promoter-*FEN1* (**a**-**c**) and *NifH* promoter-*Azotobacter vinelandii NifV* (**d**-**f**). **a**, **d**, Plants inoculated with *M. loti* TONO carrying *FEN1* (**a**) and *A. vinelandii NifV* (**d**) grown with nitrogen-free media. The wild-type Gifu and the *fen1-1* were inoculated with three independent transformants each (No. 17, 23, and 28 for *FEN1* and No. 5, 6, and 7 for *A. vinelandii NifV*, respectively). Scale bars = 10 mm. **b**, **e**, Acetylene reduction activity (ARA) of nodules formed by inoculation with *M. loti* carrying *FEN1* (**b**) and *A. vinelandii NifV* (**e**) on the wild-type Gifu (open bars) and *fen1-1* (black bars). The data are shown as means of 12 plants with standard errors. **c**, **f**. Detection of FEN1-myc (**c**) and *A. vinelandii* NIFV-myc (**f**) proteins in bacteroids isolated from the nodules formed by inoculation with *M. loti* transformants.

Figure 4 Effect of supplying homocitrate to the *fen1* mutant. **a**, Nodules formed on the wild-type Gifu and the *fen1-1* roots 4 days of incubation in the culture media supplemented with 1 mM homocitrate. After 4-days culture, some of the *fen1-1* nodules turned to exhibit red colour (indicated by arrows). Scale bar = 2

mm. **b**, Acetylene reduction activity (ARA) of nodulated roots of the wild-type Gifu and the *fen1-1* mutant 4 days after supplement with homocitrate. The data are means of five independent plants with standard errors. **c**, Detection of nitrogenase component-I and component-II in bacteroids isolated from nodules of the wild-type Gifu and from the *fen1-1* nodules supplied with homocitrate (*fen1*+HC).

Online methods and references

Plant cultivation

Seeds were surface-sterilized and sown in sterilized vermiculite. *M. loti* that had been cultured on yeast extract-mannitol-agar medium was inoculated to the seedlings. The plants were grown using nitrogen-free nutrient solution in a greenhouse or in a controlled environment growth chamber[4].

Phenotypic analyses

Nodules were fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium phosphate (pH 7.2) overnight at 4°C. The fixed nodules were dehydrated in an ethanol series and embedded in Teknovit 7100 (Kulzer) according to the manufacturer's instruction. Thin sections were made using an ultramicrotome (UltraCut-R, Leica Microsystems) with a glass knife and stained with toluidine-blue. Nitrogenase activity was assayed by acetylene reduction assay. In brief, nodulated roots were placed in 12–35-mL vials containing 10% (v/v) acetylene and incubated at 25ºC for 30 min. The amounts of ethylene produced were determined by gas chromatography[29].

cDNA cloning

The *FEN1* cDNA was amplified from RNA isolated from the Gifu nodules using a SMART RACE cDNA amplification kit (TakaRa) with 5' RACE and 3' RACE primers (Supplementary Table 1). Full-length *FEN1* cDNA was obtained by ligation of both 5' and 3' RACE products. cDNAs for *GmN56* and *AtIPMS2* were amplified by reverse transcriptase/polymerase-chain reaction (RT-PCR) from RNA isolated from soybean nodules and *Arabidopsis* leaves, respectively, using SuperScript II reverse transcriptase (Invitrogen) and Expand-High-Fidelity DNA polymerase (Rosch), with the primer sets designed from published sequences. *AtIPMS2* was cloned without the predicted N-terminal targeting sequence[9]. All the primer sequences used in this study hereafter are shown in Supplementary Table 1.

Complementation of the *fen1* mutant

The *FEN1* promoter (2 kb upstream of the translation start) and the *FEN1* terminator (1 kb downstream of the stop codon) fragments were amplified from wild-type Gifu genomic DNA by PCR. The entire *FEN1*, *GmN56* or *AtIPMS2* cDNA was inserted between them, followed by ligation into a binary vector, pC1300GFP[31]. These constructs were transformed into *A. rhizogenes* LBA1334, and then introduced into the *fen1-1* mutant by the hairy root transformation procedure as described[32].

Expression analyses

Northern blot analyses were carried out as previously described[33]. For assay of the promoter activity, the amplified *FEN1* promoter and terminator fragments were inserted into pC1300GFP, and then a Gateway vector conversion cassette (Invitrogen) was inserted between the promoter and terminator fragments. The *gusA* gene (Invitogen) was inserted into the cassette by LR clonase II (Invitorgen), to construct the *FEN1* promoter-*gusA* fusion gene (p*FEN1*-*gusA*-t*FEN1*). The fusion gene was introduced into Gifu by *A. rhizogenes*-mediated hairy root transformation[32]. The nodules formed on

transgenic roots were embedded in 5% agar and sectioned at 100 µm thickness using a microslicer (DTK-1000, Dohan EM), followed by incubation for 10–16 h in a staining solution (2 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 100 mM sodium phosphate pH 7.0). The stained sections were observed with a light microscope.

Complementation of *S. cerevisiae* mutants

The coding region for *FEN1* was amplified as described above. The mutated *FEN1* gene containing a single nucleotide mutation (*FEN1m*, corresponding to mutation in *fen1-1*) was amplified from a mixture of two overlapping DNA fragments, which were amplified by Fen1 forward primer and Fen1m internal reverse primer, and Fen1m internal forward primer and Fen1 reverse primer. The amplified PCR fragments and *AtIPMS2* cDNA were ligated into pYES2 (Invitrogen) yeast expression vector containing *GAL1* promoter. The resultant constructs were introduced into a *S. cerevisiae* IPMS mutant YMRX-3B[8] and a HCS mutant 27T6d[10]. Transformants were selected by uracil prototrophy. The production of recombinant proteins was induced by incubation at 25ºC with addition of galactose. The *S. cerevisiae* cells were collected by centrifugation and were broken with glass beads in 50 mM phosphate buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride. IPMS activity was assayed by an end point assay based on the determination of coenzyme A[9]. Homocitrate in the transformed *S. cerevisiae* cells were determined by LC/MS/MS as described below.

Determination of 2-oxoglutarate and homocitrate

Organic acids in nodules, roots and shoots were extracted with 70% ethanol. After the removal of ethanol by evaporation, the extracts were passed through a Dowex 50 column and then loaded onto a Dowex 1 column. The Dowex 1 column was washed with 15 mL water, and the carboxylic acids were eluted with 15 mL of 6 M formic acid.

After evaporation, the samples were dissolved in water. 2-Oxoglutalate was analyzed with HPLC using two anion-exclusion columns (Shodex RSpak KC-811, 8.0 × 300 mm, Showa Denko K.K.) connected tandemly at 60ºC with 3 mM perchloric acid solution (pH 2.1) as an effluent (1 mL per min). Peaks of organic acids were detected by a post-column bromothymol blue method at the wavelength of 440 nm. Homocitrate was measured with API 3000 LC/MS/MS system (Applied Biosystems/MDS analytical Technologies) using selected reaction monitoring. Samples were analyzed in the negative ion mode. Samples were loaded by connecting the mass spectrometer with a HPLC (Nanospace SI2, Shiseido Co. LTD) equipped with an ODS column (Sunfire C18 3.5 μm, 2.1 × 150 mm, Waters) using acetonitrile with 0.1% formic acid as an elution solvent. HPLC was run at a flow rate of 0.18 mL per min. Deprotonated molecule peaks ($[M-H]^-$=187) were fragmented further by CID, with N_2 as collision gas, and monitored two fragment peaks of *m/z*=125 and *m/z*=99.

Transfomation of *M. loti*

The coding region for *FEN1* was amplified by PCR from cDNA with Fen1 ORF forward and reverse primers. *NifH* promoter fragment was amplified from *M. loti* TONO genomic DNA with pNifH F and pNifH R1 primers. The 3x *myc* tag sequence was synthesized by primer extension and amplified with 3x myc F1 and 3x myc R primers. These three fragments were fused by PCR with pNifH F and 3x myc R primers, resulting in the p*NifH-FEN1*-3x *myc* fragment. The coding region for *Azotobacter vinelandii NifV* was amplified by PCR from the genomic DNA with NifV ORF forward and reverse primers. The 3x *myc* tagged *NifV* gene with *NifH* promoter fragment was constructed by fusion of the PCR fragments amplified by pNifH F, pNifH R2, 3x myc F2 and 3x myc R primers. The transposon plasmid, pCAM120[34], was modified by replacing a *Not* I fragment containing p*aph-gusA-ter* cassette with a multi-cloning site of pBluescript II SK+ with a *trpA* terminater sequence. The *FEN1* or *NifV* fragment

with *NifH* promoter described above was inserted into the modified pCAM120 and the resultant plasmid was introduced into *M. loti* by tri-parental mating with pRK2013 as a helper plasmid. Bacteroids were isolated from nodules formed by inoculation with transformed *M. loti* as described previously[35]. For immunodetection of FEN1-myc and *A. vinelandii* NIFV-myc proteins, the isolated bacteroids were suspended in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 50 mM DTT, 0.1% BPB) and subjected to SDS-PAGE with 10% (w/v) polyacrylamide gel. The proteins were blotted onto Immobilon-P filter (Millipore) and reacted with anti c-myc antibody (1:2,000 dilution; A-14, Santa Cruz biotechnology, Inc.). Immnunoreactive protein was visualized by using the ECL plus western blotting detection system (GE healthcare).

Incubation of the plants with homocitrate

The plants inoculated with *M. loti* were grown in sterilized vermiculite supplied with 1/2 strength B&D medium containing 0.5 mM potassium nitrate. Ten days after inoculation, the plants were transferred to 1/2 strength B&D medium containing 1 mM homocitrate and grown hydroponically for 4 days. Bacteroids were isolated from nodules as described previously[36], and their soluble protein fractions were obtained by sonication and centrifugation[29]. The proteins were subjected to Western blotting analysis with *Rhizobium leguminosarum* anti-nitrogenase component-I and component-II, using the ProtoBlot immunoblotting system (Promega)[29].

Supplementary References

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36. Kouchi, H., Fukai, K. & Kihara, A. Metabolism of glutamate and aspartate in bacteroids isolated from soybean root nodules. *J. Gen. Microbiol.* **137**, 2901–2910 (1991).

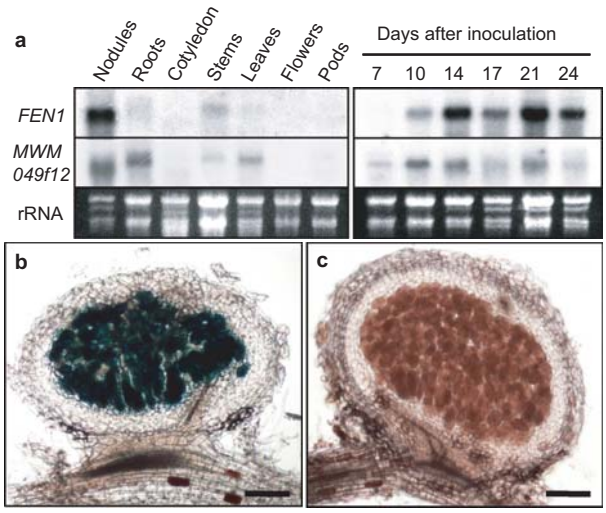
a
Nodules
Roots
Cotyledon
Stems
Leaves
Flowers
Pods
Days after inoculation
7
10
14
17
21
24
FEN1
MWM 049f12
rRNA
b
c

Figure 1

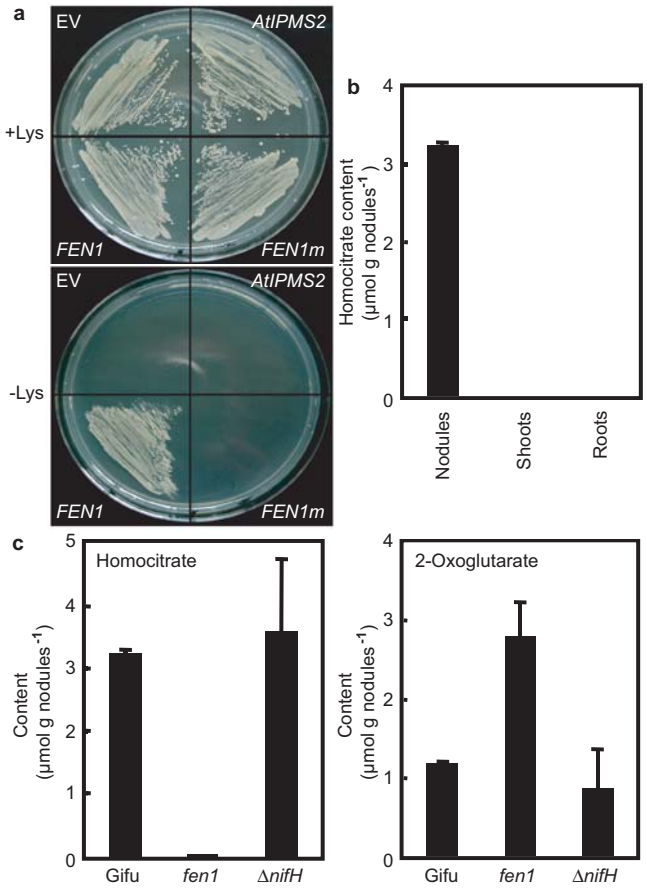
a
EV
AtIPMS2
+Lys
FEN1
FEN1m
EV
AtIPMS2
-Lys
FEN1
FEN1m
b
Homocitrate content
(µmol g nodules-1)
0
1
2
3
4
Nodules
Shoots
Roots
c
Homocitrate
Content
(µmol g nodules-1)
0
1
2
3
4
5
Gifu
fen1
ΔnifH
2-Oxoglutarate
Content
(µmol g nodules-1)
0
1
2
3
4
Gifu
fen1
ΔnifH

Figure 2

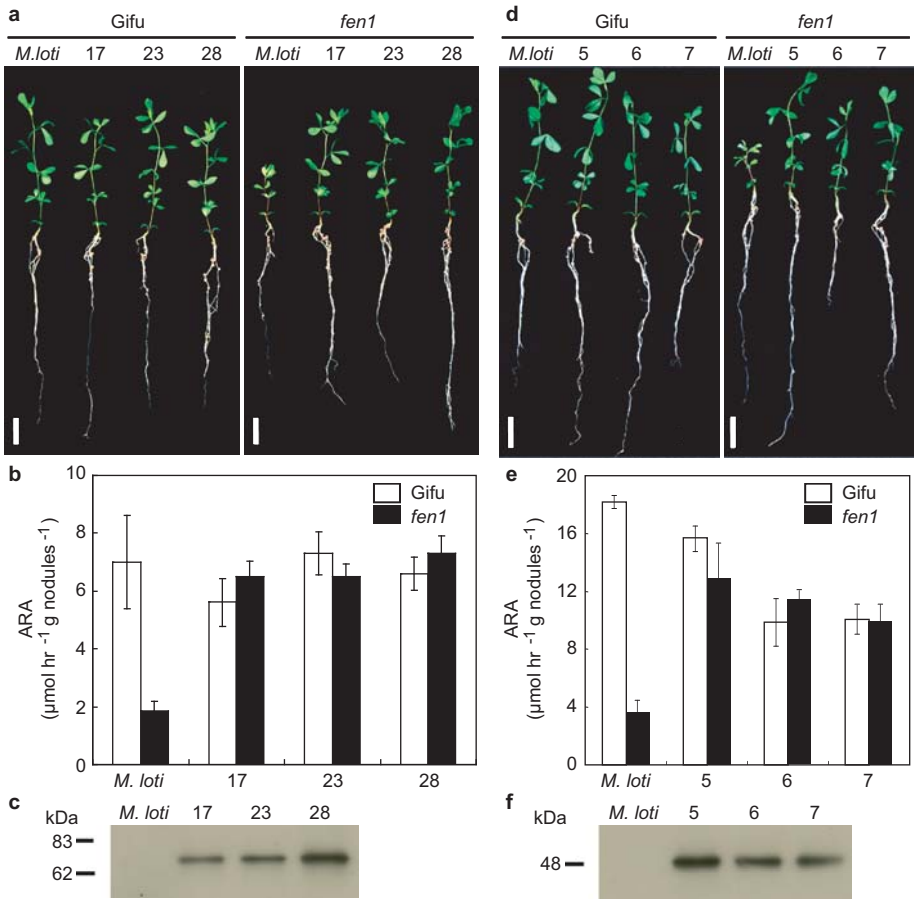
a
Gifu
fen1
M.loti 17 23 28
M.loti 17 23 28
d
Gifu
fen1
M.loti 5 6 7
M.loti 5 6 7
b
ARA
(µmol hr -1 g nodules -1)
Gifu
fen1
M. loti 17 23 28
e
ARA
(µmol hr -1 g nodules -1)
Gifu
fen1
M. loti 5 6 7
c
kDa
M. loti 17 23 28
83
62
f
kDa
M. loti 5 6 7
48

Figure 3

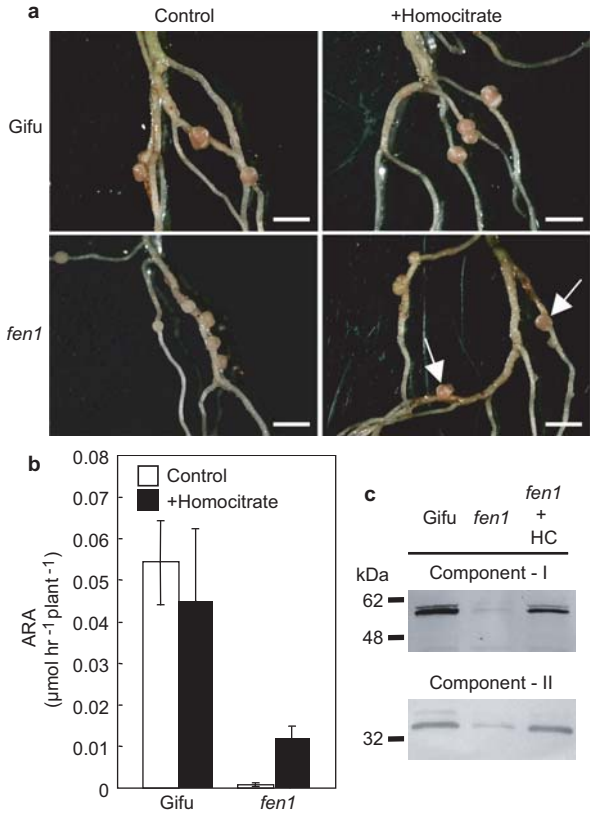
a
Control
+Homocitrate
Gifu
fen1
b
Control
+Homocitrate
ARA
(µmol hr -1 plant -1)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Gifu
fen1
c
Gifu
fen1
fen1
+
HC
kDa
Component - I
62
48
Component - II
32

Figure 4